REMARKS

The Final Office Action mailed October 20, 2004, has been received and reviewed. Claims 16-23, 25, and 27-30 are currently pending in the application. Claims 16-23, 25, and 27-30 stand rejected. Applicants propose to amend claims 16, 28, 29, and 30, and respectfully request reconsideration of the application as proposed to be amended herein.

The amendments to claims 16, 28, 29, and 30 should be entered by the Examiner because they place the application in condition for allowance. Alternatively, the amendments should be entered because they place the application in better form for appeal.

Applicants have submitted herewith copies of four previously cited references for the file history of the present application which did not accompany the PTO Form 1449 mailed January 29, 2002.

35 U.S.C. § 112 Claim Rejections

Claims 16-23, 25, and 27-30 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was allegedly not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Applicants propose to amend independent claims 16, 28, 29, and 30 to recite that the organic composition comprises at least one component selected from the group consisting of acylglycerols, fats, and oils. As such, the rejection should be withdrawn.

ENTRY OF AMENDMENTS

The proposed amendments to claims 16, 28, 29, and 30 above should be entered by the Examiner because the amendments are supported by the as-filed specification and drawings and do not add new matter to the application. Further, the amendments do not raise new issues or require a further search. Finally, if the Examiner determines that the amendments do not place the application in condition for allowance, entry is respectfully requested upon filing of a Notice of Appeal herein.

CONCLUSION

Claims 16-23, 25, and 27-30 are believed to be in condition for allowance, and an early notice thereof is respectfully solicited. Should the Examiner determine that additional issues remain which might be resolved by a telephone conference, he is respectfully invited to contact Applicants' undersigned attorney.

Respectfully submitted,

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Date: 2 DEC 2004

Methanolysis of Seed Oils in Flowing Supercritical Carbon Dioxide

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ABSTRACT: The direct methanolysis of triglycerides in flowing supercritical carbon dioxide by an immobilized lipase is described. The reaction system consists of two syringe pumps for substrate addition and another two syringe pumps for delivering CO_2 at 24.1 MPa. Corn oil is pumped into the carbon dioxide stream at a rate of 4 μL/min, and methanol is pumped at 5 μL/min to yield fatty acid methyl esters (FAME) at >98% conversion. Direct methanolysis of soy flakes gives FAME at similar yields. This combined extraction/reaction is performed at 17.2 MPa and 50°C. The fatty acid profiles obtained for these seed oils matches those obtained by classical chemical synthesis.

JAOCS 73, 353–356 (1996).

KEY WORDS: Fatty acid methyl esters, immobilized lipase, lipase, methanolysis, supercritical carbon dioxide.

The use of biocatalysts in supercritical carbon dioxide has been growing rapidly in recent years (1,2). Biocatalysts have the advantage of substate specificity under mild reaction conditions and supercritical carbon dioxide has several advantages over organic solvents (3). The solvent properties of supercritical carbon dioxide are readily modified by adjusting pressure or temperature; the diffusivity of substrates in carbon dioxide is higher than in organic solvents; carbon dioxide can easily be removed from the reaction products minimizing the need for costly downstream cleanup; when carbon dioxide is used in lieu of organic solvents, it has the additional benefit of being environmentally benigh.

Lipases in particular are amenable to syntheses in supercritical carbon dioxide. As in organic solvents (4), lipases in supercritical carbon dioxide catalyze the synthesis of esters from a variety of acids and alcohols (5–8). Lipases have been extensively applied in triglyceride technology (9–12). Lipase-catalyzed triglyceride reactions which have been carried out in supercritical carbon dioxide include interesterifications (13,14), transesterifications (15), and alcoholysis (16,17).

Fatty acid esters have a variety of uses, including antifriction agents, food preservatives, emulsifiers, and fuel alternatives. Methyl esters have been widely investigated for use as diesel fuel additives or substitutes. The large volume synthesis of fatty acid methyl esters (FAME) is accomplished by the methanolysis of fats and oils using sodium methoxide catalyst and excess methanol (18). Separation of FAME from glycerol and methanol occurs in a settling tank.

The power of supercritical carbon dioxide as a lipid solvent and reaction medium and the availability of commercial immobilized lipases present the possibility of large-scale production of FAME by means of biocatalysis. Efficient production of FAME by this approach requires a lipase with broad substrate specificity (i.e., no positional specificity) and a flow system that can run continuously. A lipase isolated from *Candida antarctica* and immobilized on polyacrylamide has been found to be an appropriate catalyst for this purpose. A variety of ester syntheses have been catalyzed by this enzyme (19–22). We have also found that it is quantitative with respect to catalyzing methanolysis of a variety of natural triglycerides of different fatty acid compositions.

There are only a few reports of syntheses in flowing supercritical carbon dioxide (23–25). This paper describes a continuous-flow bioreactor and the conditions used for the conversion of soybean and corn oils to FAME.

MATERIALS AND METHODS

Corn oil was purchased at a local grocery. Soy flakes were prepared by the method of Galloway (26). Carbon dioxide sources were from National Welding Supply (welding grade used in the soy flake methanolysis; Bloomington, IL) and Air Products (analytical grade; Allentown, PA). High-performance liquid chromatography (HPLC)-grade methanol from Fisher Chemicals (Fairlawn, NJ) was used without further purification. Novozym 435 was obtained as a generous gift from Novo Nordisk (Danbury, CT). The immobilized enzyme is described by the manufacturer as containing 1—2% water by weight and having 7000 units/g toward propyl laurate.

Carbon dioxide was pumped with Isco, Inc. (Lincoln, NE) 100 DX syringe pumps, cooled to −10°C, and set up in a continuous-flow mode. The substrates were also pumped with Isco 100 DX pumps as shown in Figure 1.

Reactions were performed at 24.1 MPa, 50°C, at a corn oil flow of 4 μL/min. The restrictor temperature was set at 50°C to maintain a supercritical CO_2 flow of 1.0 mL/min. A steady state was established after all pressures and flows through the

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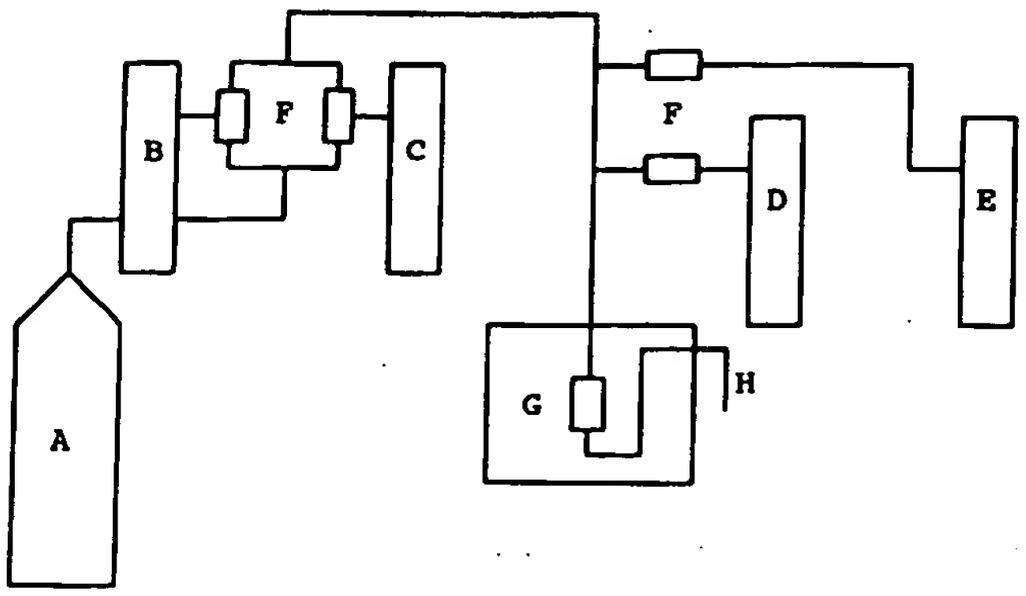

FIG. 1. Schematic of the continuous-flow system used for methanolysis of corn oil. A, CO_2 tank; B and C, CO_2 pumps; D, methanol pump; E, corn oil pump; F, check valves; G, enzyme bed in oven; H, restrictor.

system stabilized. This typically required 30 min. Products were collected in an open test tube immersed in a dry ice/isopropyl alcohol bath. Recoveries were in the range of 85–95%.

The effect of water on the transesterification was studied by flowing the CO_2 over water-saturated glass wool, inserted before the catalyst, and by adding known volumes of water to the methanol. The aqueous methanol solution was pumped at rates that maintained a methanol flow of 5 µL/min.

Methanolysis of soy flakes was performed in an apparatus represented by the schematic in Figure 2. Methanol was added with an HPLC pump (Model 100a, Beckman Instrument Inc., Fullerton, CA). The extraction vessel and the enzyme bed were connected in series as shown. The soy flakes were lyophilized prior to methanolysis in an FTS Systems Flexi-dry freeze dryer (Stone Ridge, NY). Final water content of the flakes was 2% by weight. Oil content of the flakes was 20% by weight. For the methanolysis, about 16 g flakes were placed in a stainless-steel cell (1.7 × 23 cm) and held in place with glass wool plugs. Novozym 435 (1.4 g) was placed in a separate stainless-steel vessel (0.8 × 10.2 cm) downstream from the flakes. The system was heated to 50°C and purged with CO_2 at 5.5 MPa while methanol was pumped into the system

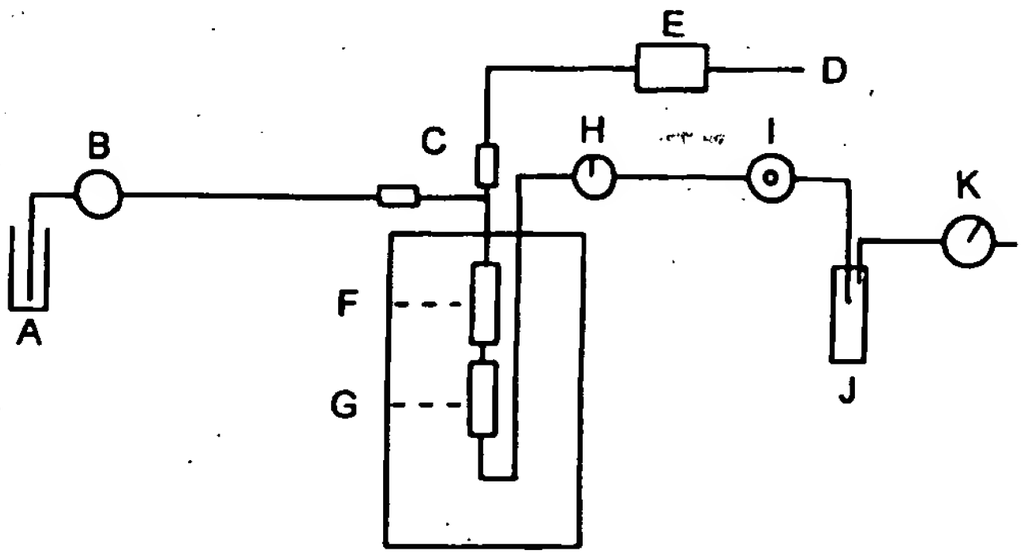

FIG. 2. Schematic of the system used for methanolysis of soy flakes. A, methanol source; B, high performance liquid chromatography pump; C, check valves, D, CO_2 source; E, gas booster; F, soy flakes and G, enzyme bed in oven; H, pressure gauge; I, micrometering valve; J, receiver; K, gas meter.

(10 µL/min). The CO_2 pressure was then increased to 17.2 MPa with a gas booster pump (Model AC-30-C; Haskel Mfg. Co., Burbank, CA). Flow was set at 8 L/min by a micrometering valve (Series 30VRMM; Autoclave Engineers, Erie, PA) as measured as expanded gas by a dry test meter.

Products were separated on a Lee Scientific Series 600 SFC/GC (Dionex, Inc., Salt Lake City, UT) with a Dionex SB-Octyl-50 column (10 m × 100 µm i.d. × 0.5 µm film). The density gradient was as follows: temperature: 100°C, 5 min, then 8°/min to 190°C; pressure: 120 atm, 5 min, then 8 atm/min to 300 atm. Samples were injected with a Valco (Valco, Inc., Houston, TX) injection loop (200 nL) held open for 1.8 s. Analytes were detected by an flame-ionization detector (FID) operating at 350°C. Conversion was determined by the ratio of the methyl ester peak area to the triglyceride peak area.

FAME were analyzed on a Hewlett-Packard 5890 Series II gas chromatograph with electronic pressure control. Instrument settings were: injector, 235°C; FID, 250°C; column head pressure, 140 kPa; carrier gas (helium) flow, 1 mL/min. FAME were separated on a Supelco (Bellefonte, PA) SP-2340 column (60 m × 0.25 mm i.d., 0.2 µm film). Chemical derivatization was accomplished by the BF_3-MeOH method (27). Glycerol was determined semiquantitatively by reversed-phase (RP) HPLC with an FID (28) and by spot test analysis (29).

RESULTS AND DISCUSSION

Methanolysis of corn oil. A schematic of the flow system used to produce FAME from corn oil is shown in Figure 1. Conversions were performed at 24.1 MPa and 50°C, conditions consistent with the maintenance of enzyme activity. This corresponds to a CO_2 density of 0.83 g/mL. Corn oil was pumped at a flow of 4 µL/min when CO_2 flow was at 1 mL/min. At higher flow of corn oil or lower pressures, oil precipitates onto the exterior walls of the reaction cell; therefore, reactions were performed at the above conditions.

In a typical run, 425 mg corn oil was pumped over 500 mg Novozym 435 to yield 365 mg product. The product was usually >95% FAME, with the balance of products being mono-, di-, and triglycerides. Upon standing at room temperature, colorless crystals precipitated out of the FAME solution. RP-HPLC determined that these crystals were monoglycerides.

Characterization of methyl ester products. The fatty acid composition of corn and soybean oils as determined by chemical derivatization (BF_3/methanol) and by methanolysis with Novozym 435 is presented in Table 1. The FAME profiles are identical, regardless of the derivatization method used, indicating that the methanolysis as catalyzed by Novozym 435 was nonspecific and complete.

Isolation of glycerol. It was anticipated that the low solubility of glycerol in supercritical carbon dioxide might inhibit methanolysis, due to precipitation of glycerol onto the enzyme bed. However, only a small amount of glycerol was ever found on the catalyst. Analysis by RP-HPLC showed

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SUBTILISIN-CATALYZED TRANSESTERIFICATION IN SUPERCRITICAL CARBON DIOXIDE

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SUMMARY

Subtilisin Carlsberg was found to catalyze transesterification between N-acetyl-L-phenylalanine chloroethyl ester and ethanol in supercritical carbon dioxide. The effects of different temperatures and carbon dioxide/ethanol ratios on the reaction rate were investigated. A comparative study showed that enzymatic transesterification is faster in supercritical carbon dioxide than in anhydrous organic solvents.

INTRODUCTION

It has been shown by many investigators (see review articles: Klibanov, 1989; Khmelnitsky et al., 1988: Dordick, 1989) that enzymatic catalysis in nearly anhydrous organic solvents offers important advantages over aqueous buffers in the transformation of hydrophobic compounds and the synthesis of amide and ester bonds. Recently, supercritical carbon dioxide has also been employed as a medium for reactions catalyzed by alkaline phosphatase (Randolph et al., 1985), polyphenol oxidase (Hammond et al., 1985), cholesterol oxidase (Randolph et al., 1988) and lipases (Van Eijs et al., 1988; Nakamura et al., 1988).

Supercritical carbon dioxide (critical temperature 31.1°C), which is non-toxic and easily removable, is a good solvent for hydrophobic compounds and gases and affords high mass transfer and diffusion rates (Schneider

et al., 1980; Smith, 1988).

Previously, subtilisin Carlsberg was found to retain its catalytic activity when suspended in organic solvents ranging from hydrophobic hexane to water-miscible acetone and dimethylformamide (Zaks and Klibanov, 1988; Riva et al., 1988; Carrea et al., 1989). In the present study, we have established that this enzyme can also be used in supercritical carbon dioxide. The model transesterification reaction between N-acetyl-L-phenylalanine chloroethyl ester and ethanol (Fig. 1) was carried out at different carbon dioxide/ethanol ratios and temperatures and the results compared with those obtained in organic solvents.

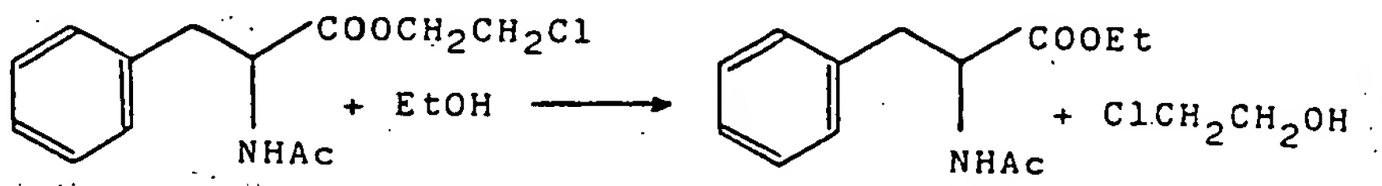

Fig 1. Enzymatic transesterification between N-acetyl-L-phenylalanine chloroethyl ester and ethanol to give N-acetyl-L-phenylalanine ethyl ester and chloroethanol.

MATERIALS AND METHODS

Materials. Subtilisin Carlsberg was obtained from Sigma and dissolved in water prior to use, adjusted to pH 7.8 and freeze-dried (Riva et al., 1988). Carbon dioxide was obtained from S.I.A.D.. N-acetyl-L-phenylalanine chloroethyl ester was synthesized as previously reported (Riva et al., 1988) and the standard N-acetyl-L-phenylalanine ethyl ester was bought from Sigma. All other reagents and compounds were of analytical grade.

Analysis. The transesterification reaction was followed by gas chromatography with a 25 m HP1 capillary silica gel column coated with methylsilicone gum (Hewlett Packard) (hydrogen carrier gas 30 ml/min; oven temperature 250°C). Retention times for N-acetyl-L-phenylalanine chloroethyl ester and N-acetyl-L-phenylalanine ethyl ester were 5.7 and 4.2 min.

Transesterification in organic solvents. Subtilisin (10 mg) was added to 1 ml of an organic solvent containing 56 µmol (15 mg) of N-acetyl-L-phenylalanine chloroethyl ester and 2-5 % (v/v) ethanol and the suspension shaken at 250

rpm in an orbital shaker, at 45°C. The reaction was followed by gas chromatography.

Transesterification in supercritical carbon dioxide. A Jasco Super-200 SFE (SFE, supercritical fluid extraction) system was employed (Fig.2). It consisted of a thermostated reaction vessel, two HPLC pumps (mod. 880-PU) and a back pressure regulator valve (mod. 880-81) able to control outlet pressure up to 300 bar independently of the mass flow rate of the fluid. Liquefied carbon dioxide was pumped at a rate of 2 ml/min and mixed with ethanol which was pumped at the rate of 0.01-0.1 ml/min. Subtilisin (10 mg), 56 μmoles of substrate and a small magnetic bar were placed in the reactor (1 ml volume) which was in contact with a magnetic stirrer. Before filling the reaction vessel, the hydraulic system was equilibrated with the required ratio between carbon dioxide and ethanol (V1 valve was open and V2 and V3 valves were closed). At zero time the V2 valve was opened allowing the supercritical fluid to fill the vessel. In that position the reaction vessel was in a dead arm of the circuit but still kept under the set pressure and composition. After a scheduled time the V1 valve was closed and the V3 valve was opened and the material in the vessel was transferred to the fraction collector and analyzed by gas chromatography.

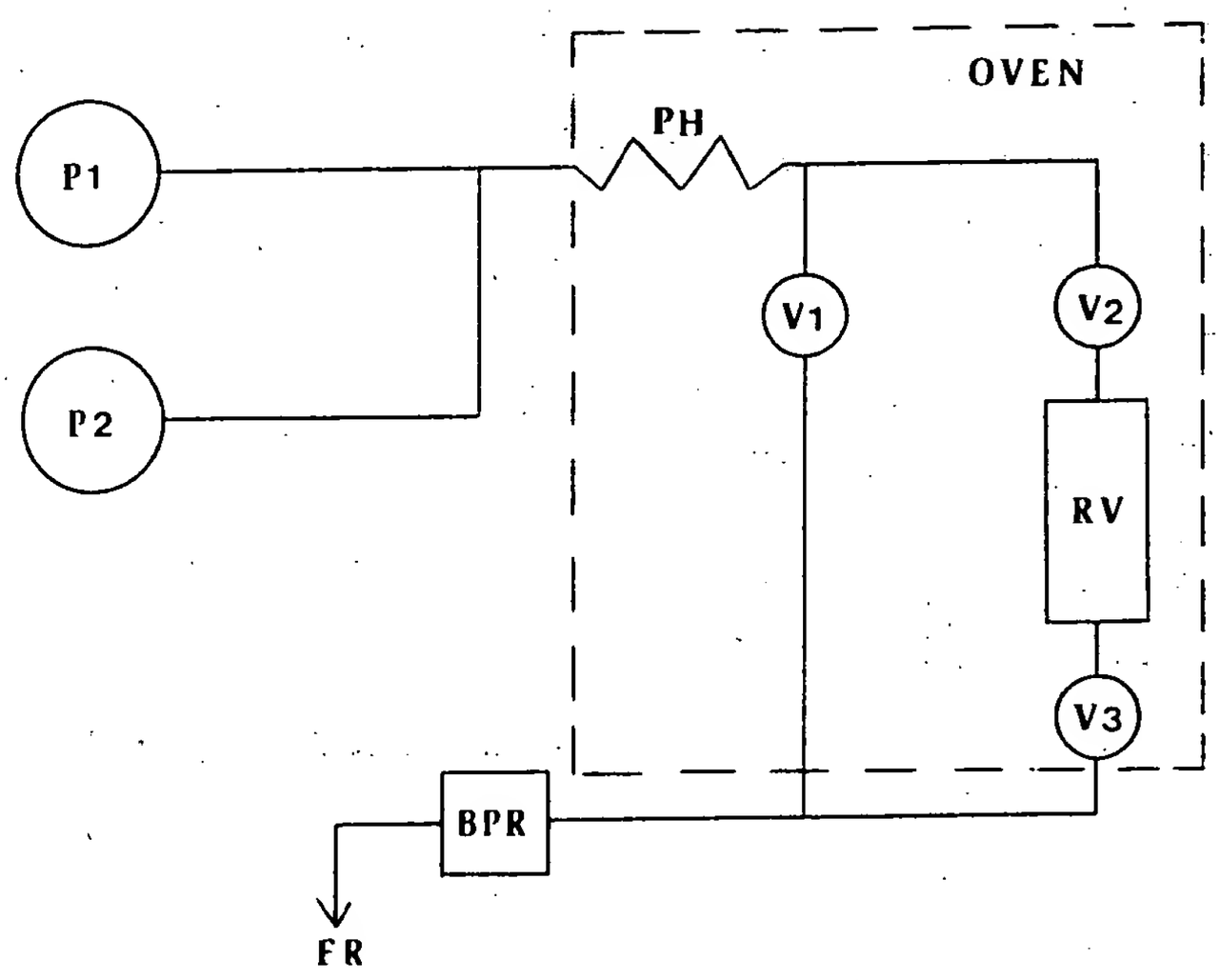

Fig.2. Hydraulic diagram of SFE preparative system. Components: P1=carbon dioxide pump with cooling jacket; P2=modifier solvent pump ; PH=preheating coil, 0.5 mm i.d. x 5 m length; V1,V2,V3= valves; RV=reaction vessel, 1 ml; BPR=back-pressure regulator; FR=fraction collector.

RESULTS AND DISCUSSION

Transesterification between N-acetyl-L-phenylalanine

chloroethyl ester and ethanol (Fig.1) in supercritical carbon dioxide was carried out at 45°C and 150 bar, using concentrations of ethanol ranging from 0.5 to 5% (v/v) and a reaction time of 15 min. Conversions were 10 to 54%, with the highest value obtained at the intermediate concentration of 2.5% ethanol. The reaction in 2.5% ethanol is shown in Fig.3, where it can be seen that the transformation was almost complete after 30 min reaction. The recoveries of product plus untransformed substrate from the reactor were ≥ 90% .

Control experiments showed that there was no modification of the substrate in the absence of ethanol and that bovine serum albumin or subtilisin inactivated with phenylmethanesulfonyl fluoride (Zaks and Klibanov, 1988) caused negligible transesterification. Therefore, the observed transesterification in supercritical carbon dioxide is totally a subtilisin-catalyzed reaction.

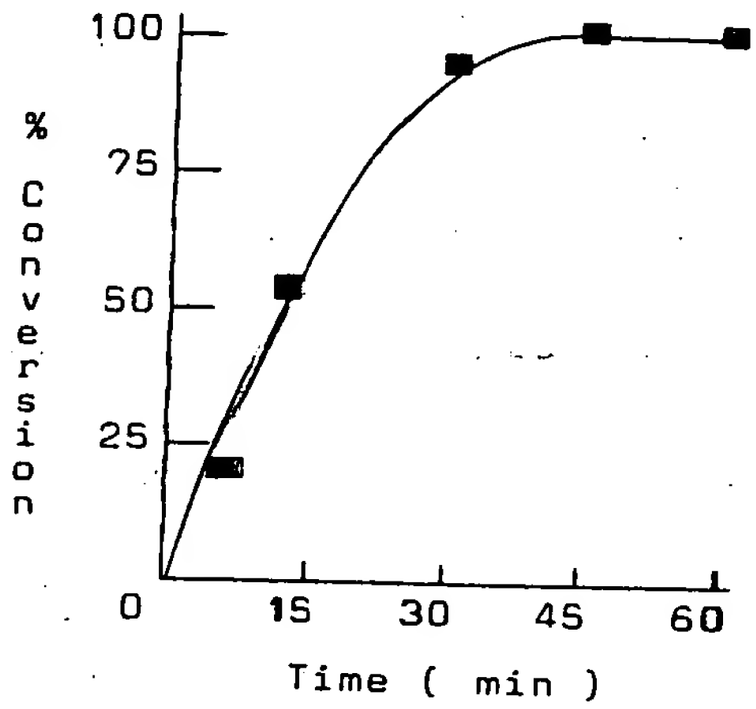

Fig.3. Conversion of N-acetyl-L-phenylalanine chloroethyl ester to N-acetyl-L-phenylalanine ethyl ester in supercritical carbon dioxide as a function of time. Conditions: 10 mg of subtilisin ; 56 µmol of substrate; ethanol in carbon dioxide, 2.5 % ; 45°C; 150 bar; reactor volume 1 ml.

Subtilisin was used for repeated transformations (up to 3 cycles) without appreciable decay of activity. This good enzyme stability in supercritical carbon dioxide was further confirmed by the fact that the reaction rate increased when the temperature was increased to 80°C.

Transesterification was also carried out in some

organic solvents and the results are shown in Table 1. A comparison with the data of Fig.3 indicates that the reaction was more effective in supercritical carbon dioxide. In the most suitable solvent (tert-amyl alcohol) 89% conversion was obtained after 2 hours, compared with 92% conversion after 30 min in supercritical carbon dioxide. It should be noted that the same amounts of enzyme and substrate were used in both cases. The higher reaction rate in supercritical carbon dioxide should reflect mass transfer and diffusion rates higher than in organic solvents.

Table 1. Conversion of N-acetyl-L-phenylalanine chloroethyl ester to N-acetyl-L-phenylalanine ethyl ester in organic solvents.

	% Conversion		
	2% Ethanol		5% Ethanol
Solvent	1 hour	2 hours	2 hours
Acetone	16	27	53
Pyridine	11	20	23
tert-Butanol	--	--	67
tert-Amyl alcohol	61	75	89
Benzene	6	12	27

Another difference between the two systems regards the effects of ethanol concentration. In organic solvents the reaction rate increased between 2 and 5% ethanol whereas in supercritical carbon dioxide it decreased. Increasing concentrations of ethanol may affect the diffusivity properties of the supercritical solvent.

In conclusion, the results demonstrate that the transesterification reaction catalyzed by subtilisin is faster in supercritical carbon dioxide than in organic solvents. This, together with its non-toxicity and the easy removal of carbon dioxide, makes the system highly promising and worth a further study of how to scale it up and apply it to other enzymes and substrates.

Acnowledgements

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Kinetics of Lipase-Catalyzed Esterification in Supercritical CO_2

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This study compares two solvents for enzymatic reactions: supercritical carbon dioxide ($SCCO_2$) and organic solvent (*n*-hexane). The model reaction that was chosen was the esterification of oleic acid by ethanol catalyzed by an immobilized lipase from *Mucor miehei* (Lypozyme). The stability of the enzyme appeared to be quite good and similar in both media but was affected by the water content. Partition of water between solvents and immobilized enzyme has been calculated from experimental adsorption isotherms. The water content of the solid phase has a dramatic influence on the activity of the enzyme and its optimum value for activity was about 10% (w/w) in both media. A kinetic study enabled a Ping-Pong Bi-Bi reaction mechanism with inhibition by ethanol to be suggested. Despite some differences in kinetic constants, activity was in the same range in both media. Hypotheses for explaining the kinetic constant variations have been proposed and particular attention has been paid to the pH effects.
Key words: enzyme • supercritical CO_2 • stability • effect of water • comparison with organic solvent

INTRODUCTION

The use of enzymes acting as catalysts in nonaqueous media has been frequently described in the scientific literature.[2,11,12,20,27–30] Organic media present certain advantages such as stabilization of enzymes, dissolution of hydrophobic compounds, and the possibility of shifting thermodynamic equilibrium toward the synthesis of esters and amides (e.g., in the case of hydrolytic enzymes).

Among these media, the supercritical state of some fluids, such as carbon dioxide, may provide an interesting alternative. In the supercritical state, CO_2 exhibits properties similar to organic solvents, but with additional capacities of encouraging transport phenomena and facilitating postreactional separation (due to variable solvent power), which make it more attractive.

Enzymatic reactions in supercritical CO_2 have been scarcely studied since the work of Nakamura et al. in 1985[16] on the interesterification of triglycerides by a lipase. A few other studies are now available and have proved the feasibility of enzymatic reactions in $SCCO_2$.[4,7,9,14,18,19,24]

Elsewhere[14] we presented the first approach of the lipase catalyzed esterification of a fatty acid in supercritical carbon dioxide ($SCCO_2$) and in an organic solvent (*n*-hexane). Esterification of oleic acid by ethanol with an immobilized lipase from *Mucor miehei* was chosen as a model reaction. Results were processed by a simple Michaelis–Menten model and water content of the media was indicated as a crucial parameter.

The present work is a more complete study of the same reaction leading to the establishment of the mechanism of reaction and to an improved quantification of the water content effect through the determination of experimental data upon the thermodynamic partition of water between the enzymatic support and the fluid. A complete study of enzyme stability, including the effect of pressure and water content has also been added.

A first complete approach of the comparison of enzymatic reaction in supercritical carbon dioxide and in an organic solvent is presented.

MATERIALS AND METHODS

Materials

Immobilized lipase (EC 3.1.1.3, Lipozyme™; 200 U/g) from *Mucor miehei* supported on a macroporous anionic resin beads was kindly provided by Novo (Denmark).

Oleic acid (*cis*-9-octadecanoïc acid) with an approximate purity of 99% was supplied by Sigma, absolute ethanol and *n*-hexane from Prolabo (France), and carbon dioxide (N45) with less than 7 ppm of water from Alphagaz (France).

Analytical Methods

Oleic acid and ethyl oleate concentrations were determined by high-performance liquid chromatography (HPLC) using a Kontron 420 pump system with a Varian R14 refractive index monitor on a Nucleosil C_{18} 5-μm column (250 × 4.6 mm) (SFCC, France). Elution was conducted at 40°C with methanol/acetic acid (99.7/0.3, v/v) and a flow rate of 0.7 mL/min.

Ethanol concentration was determined by an Intersmat IGC 120 DFL gas chromatograph equipped with a flame ionization detector. Separation took place in a column length of 2 m and a diameter of 3.2 mm packed with a Porapack QS 80-100 mesh. Nitrogen was used as a carrier gas. Injector, detector, and column temperatures were 260, 260, and 195°C, respectively. Quantita-

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tive data were obtained by peak integration with an Intersmat ICR 1B after calibration with propanol 2 (5 g/L) internal standard.

Enzymatic Activities in $SCCO_2$

An original device has been conceived to provide easier handling than those used by previous workers. It consists of a sapphire tubular reactor of 16 mL volume, allowing visual control of the phases. The reactional fluid is recirculated through an external loop by means of a high-pressure gear pump. In the external loop, pressure and temperature measurements, temperature control, and a sample loop are set. This last item allows a whole kinetic curve in one single run to be obtained. The total volume is 70 mL. A schematic diagram is presented in Figure 1. The whole system is placed in a temperature-controlled room to prevent any temperature gradient. After solubilization of oleic acid in the reactor containing the immobilized enzyme, ethanol is introduced into the system by the sample loop giving the zero time of the reaction. Every sample withdrawn with the sample loop, representing 0.9% of the total volume, is flashed and then rinsed by the HPLC eluent.

Enzymatic Activities in *n*-Hexane

Ester syntheses were carried out in glass tubes containing oleic acid, ethanol, and 10 mg immobilized lipase. The reaction mixture was incubated at 40°C and agitated with magnetic stirring. Samples were withdrawn at various intervals to determine product concentrations by HPLC.

Enzymatic Stabilities in $SCCO_2$

The system, consisting of 1-mL microvessels containing the tested enzyme in supercritical conditions, was immersed in a temperature-controlled bath. Every 2 days, one vessel was disconnected and residual esterase activity was measured. Every point represents the average of two manipulations.

Enzymatic Stability in $SCCO_2$ in Presence of Water

Fifty milligrams of enzyme was introduced into a 3.8-mL reactor in the presence of variable quantities of water. The reactor was incubated at 40°C and agitated with magnetic stirring. After 1 day, it was disconnected, and the enzyme was dried with a control sample. Residual activity was measured.

Determination of Adsorption Isotherms

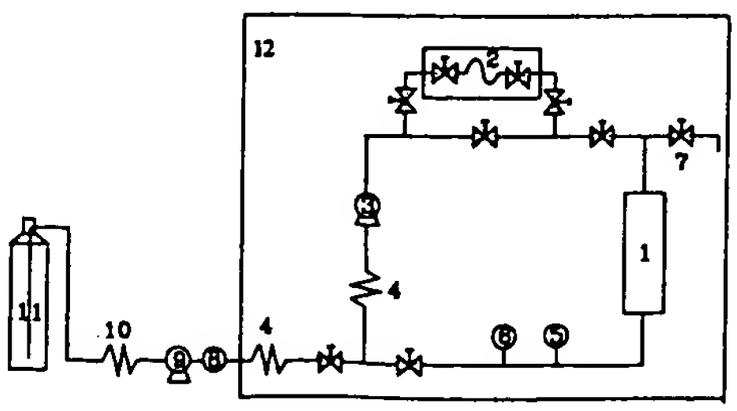
Adsorption isotherms provide a measurement of the partition of water between a solvent and the enzymatic support. The device presented in Figure 1, with an additional reactor in series with the first, was used. The immobilized enzyme located in one reactor was equilibrated with a mixture of CO_2, water, and ethanol for half an hour through the recirculation loop. This reactor filled with the immobilized enzyme was isolated and water adsorbed on the solid was assayed, after slow depressurization, by the Karl Fischer titration method.[21] Carbon dioxide of the second reactor was also slowly depressurized in ethanol (with a known water content). Finally, the water content of ethanol was measured, which leads to the water content of carbon dioxide. The Karl Fisher device consists of a T90/50 cruet automatically controlled by the titrator Schött Gerate TR 151. The mass balance for water has been checked and hence validates the experimental method.

Computational Method

Parametric identification of maximum velocity, affinity constants, and inhibition constant was used from the equation for reaction velocity. The program of identification was based on minimization of quadratic errors using a Gauss–Newton algorithm.

STUDY OF THE STABILITY IN SUPERCRITICAL CO_2

One of the interesting results of operating enzymatic catalysis in nonaqueous media is the increase in stability with respect to aqueous media. This effect has often been observed. For instance, Zaks and Klibanov[27] have presented a half-time for porcine pancreatic lipase in organic media at 100°C of about 10 h, whereas this enzyme is instantaneously inactivated in water. Taniguchi et al.[24] have studied the stability of nine commercial enzymes in supercritical CO_2. They found a quite good stability of 200 atm, 35°C, but their experiments were



-1- reaction vessel
-2- sample loop
-3- recirculation pump
-4- heater
-5-6- temperature and pressure measurements
-7- valve
-8- 200 bars max security disk
-9- high pressure pump
-10- cooler
-11- liquid CO_2 tank
-12- temperature controlled room.

Figure 1. High-pressure device for realization of enzymatic reaction in $SCCO_2$.

conducted only during 1 h. Our first study[14] had shown that immobilized lipase submitted to supercritical CO_2 exhibits a slight decay in activity after 6 days (10% loss after 6 days at 40°C, 13 MPa), and temperature has been shown to have a low negative influence (20% loss after 6 days at 60°C, 13 MPa). It was also demonstrated that stability of the enzyme in $SCCO_2$ was quite similar to stability in *n*-hexane.

Additional experiments proved that pressure has no effect on stability in the range investigated (13–18 MPa) with only 10% loss of activity after 6 days at 40°C.

Another important parameter is the water content of the immobilized enzyme, and all previously mentioned experiments have been performed with commercial enzymes containing approximately 8% water (w/w of support). In the latest series of experiments we added different amounts of water to the immobilized enzyme and measured residual activity after 1 day. Figure 2 shows residual activity of the enzyme versus added water. It presents a decrease of stability due to the presence of water in the system. Enzyme seems to be irreversibly denaturated in the presence of water. Indeed, water is suspected of leading to undesirable reactions such as hydrolysis of proteins and formation of inadequate structure.

ISOTHERMS OF ADSORPTION OF WATER BETWEEN ENZYMATIC SUPPORT AND SOLVENT

In our first study[14] we pointed out the dramatic influence of water content on enzymatic activity. In order to quantify this parameter, adsorption isotherms of water between the solvent and the enzymatic support were determined. Therefore, in the following studies, enzymatic activity will refer to water present on enzymatic support and not to water added to the reaction. Thus, it will be possible to compare activity in $SCCO_2$ with activity in *n*-hexane (these two media present significant differences of hydrophobicity) at the same water content in the vicinity of the enzyme.

The curves in Figures 3 and 4 illustrate the influence of pressure and temperature of $SCCO_2$ containing 150 m*M* ethanol.

Increasing temperature has a negative effect on adsorption of water on the solid, as may usually be expected in gas adsorption. On the other hand, increasing pressure also has a negative effect that is opposite to results of gas adsorption and means that here the solvation effect is predominant over the vapor pressure effect.

These results follow the evolution of water solubility in $SCCO_2$ as a function of pressure and temperature, as may be calculated from the equation of Chrastil.[5] Solubility is improved with an increase of pressure or an increase of temperature for pressures higher than 13 MPa (below 13 MPa it is no longer true because of the retrograde solubility phenomenon).

We also studied the influence of ethanol content of the solvent, and a suspected "entrainer effect" is visible in Figure 5 which leads to the "drying" of the enzymatic support. This last result could play a role in the interpretation of the kinetic results and will be discussed further.

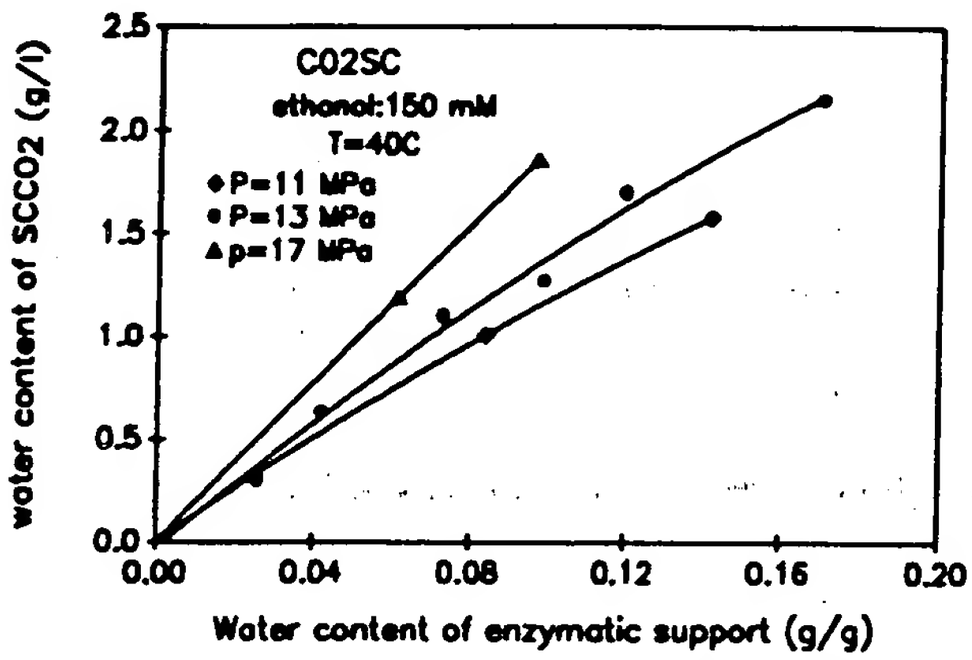


Figure 3. Partition of water between enzymatic support and $SCCO_2$ (40°C) as function of pressure.

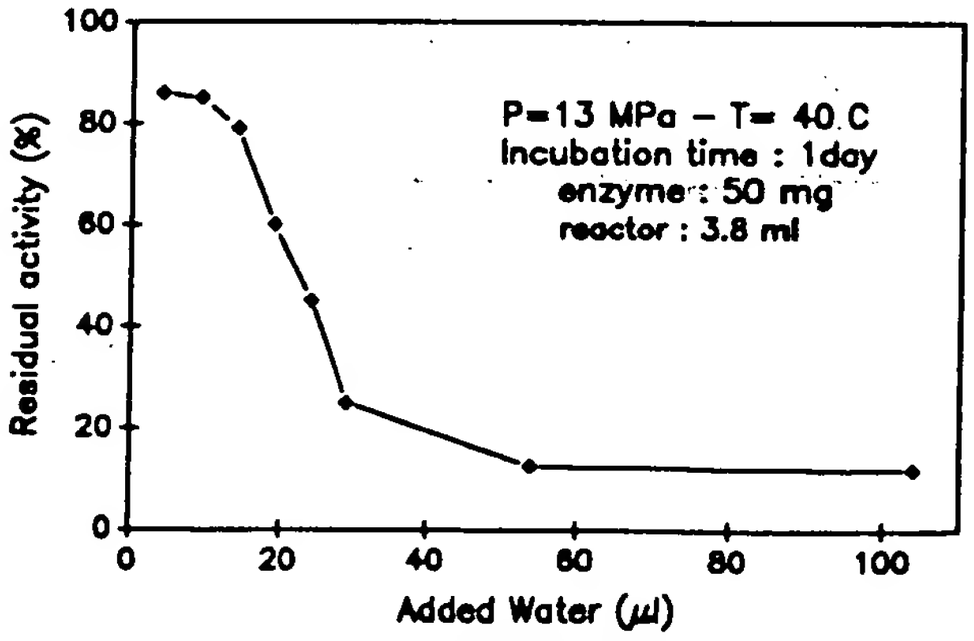


Figure 2. Residual activity in $SCCO_2$ (13 MPa, 40°C) as function of added water.

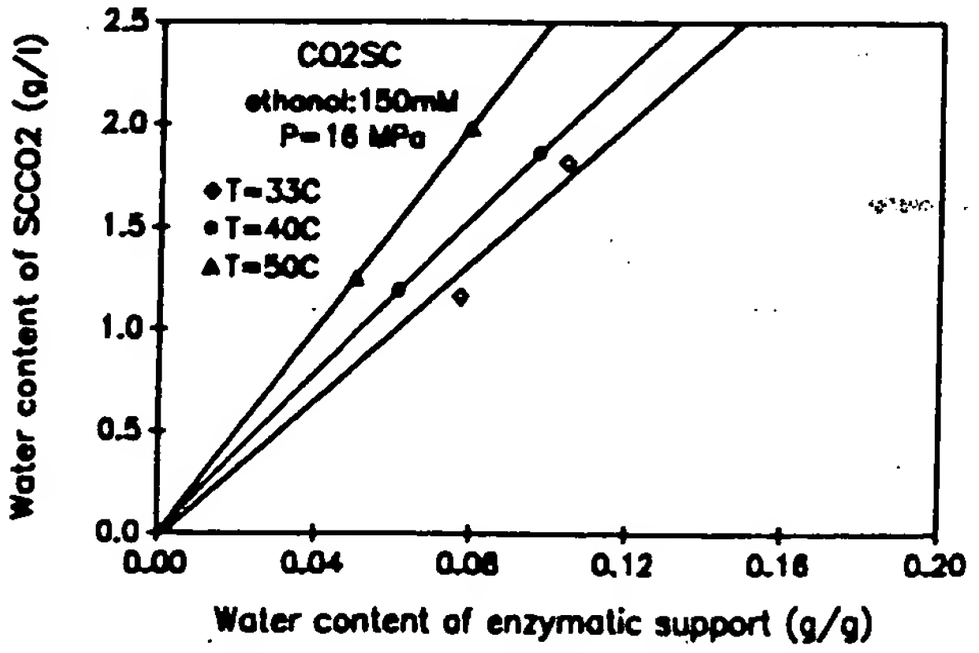


Figure 4. Partition of water between enzymatic support and $SCCO_2$ (15 MPa) as function of temperature.

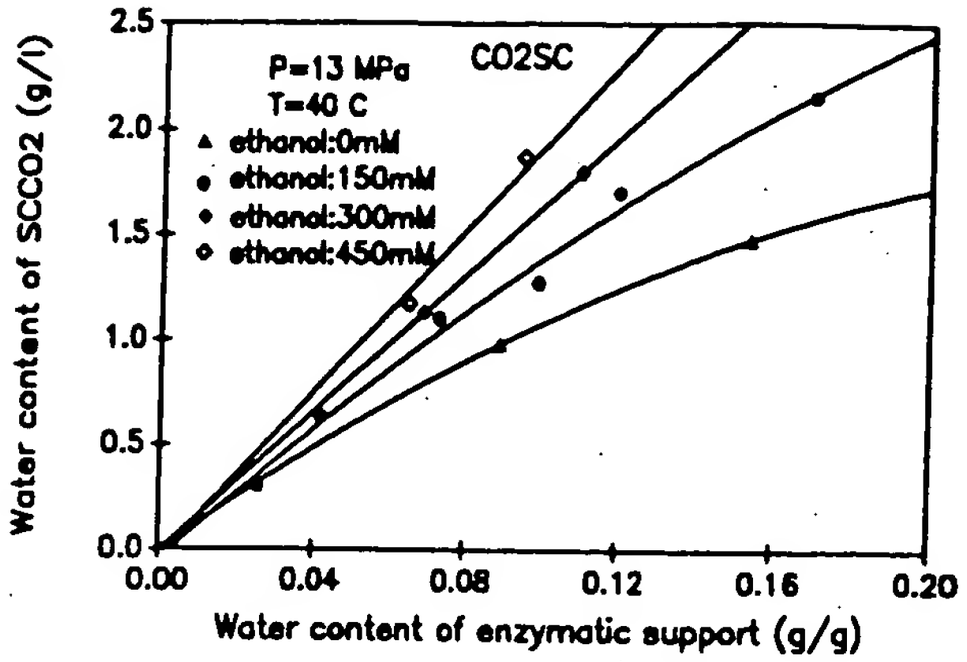


Figure 5. Partition of water between enzymatic support and $SCCO_2$ (13 MPA, 40°C) as function of ethanol concentration.

To estimate the solubility of water in the $SCCO_2$ phase, we used the empirical equation suggested by Chrastil[5] based on the concept of complex formation between the solute and the solvent molecules and on the law of mass action applied to the equilibrium between this complex and the free molecules. This equation relates solubility of a pure compound as a function of temperature and density of the SC phase.

The density of SC carbon dioxide versus pressure, temperature, and solute concentrations was calculated using PROPHY, developed by X. Joulia et al.[10] to study the thermodynamic properties and equilibrium between phases. The thermodynamic state equation was from the Lee–Kesler–Plocker model.

Calculation of water solubility in carbon dioxide without ethanol is in good agreement with experimental results given by Chrastil[5] and Wiebe and Gaddy.[26]

The influence of ethanol concentration could be deduced from studies of ethanol–water–carbon dioxide phase equilibrium published by de la Ossa et al.[17] at 40°C, 10 and 20 MPa, and by Nagahama et al.[15] at 40°C and 10 MPa. The enhancement factor of water solubility was deduced as a function of ethanol concentration at 40°C, 10 and 20 MPa. This enhancement factor was interpolated at various pressures, enabling approximate maximum water solubility in supercritical carbon dioxide to be determined (Table I).

From these results, it may be concluded that, at 0.02 g water/g enzymatic support, curves are near the water saturation of the carbon dioxide phase, except for curves at high ethanol concentrations (300 and 450 m*M*).

Partition of water is a function of the hydrophobic character of both the enzymatic support and the solvent. Comparison of adsorption isotherms in hexane (40°C) and in the supercritical fluid (13 MPa, 40°C) with 150 m*M* ethanol is presented in Figure 6. The $SCCO_2$ appears much more hydrophile than *n*-hexane. Therefore, in order to obtain the same water content in the vicinity of the enzyme in both solvents, more water has to be added in $SCCO_2$.

INFLUENCE OF WATER ON ACTIVITY

The influence of water content of the support on activity has been studied in $SCCO_2$ at 13 MPa, 40°C, and with a 150 m*M* ethanol content. The value of the water content of the support has been calculated by a mass balance between initial water content of the support, quantity of added water, and use of the experimental adsorption isotherms. Sharp, bell-shaped curves are obtained (Fig. 7) and are typical of a phenomenon that has often been found for activity of enzymes in organic solvents.[6,20] On the one hand, enzymes need a small amount of water to maintain their active conformation. On the other hand, with increasing water content negative effects on activity appear in relation to hydrophilic hindrance for the hydrophobic substrate on its way to proteins. Optimum water content of the support may be estimated at roughly 10% (w/w) whatever the operating conditions.

It is interesting to note that the same optimum of activity is obtained in both media. For these solvents with poor water solubilities, the solvent acts only by the difference in the water partition that it induces.

MECHANISM OF REACTION

This study was conducted with a 10% optimum water content of the solid at 13 MPa and 40°C, which involves the addition of more water in the supercritical fluid than in *n*-hexane. Undeniably, the water content of the solvent is different in both media. This factor influences the kinetic constants. Nevertheless, the complexity of the problem leads us to consider that the most important effect of water is to obtain the optimum enzyme conformation. Consequently, initial rate formulation does not take water influence into account in the most appropriate way.

Table I. Maximum water solubility in $SCCO_2$ in function of pressure, temperature, and ethanol concentration.

	Ethanol, 150 m*M*, 40°C			Ethanol, 150 m*M*, 16 MPa			13 MPa, 40°C with ethanol		
Conditions	11 MPa	13 MPa	17 MPa	33°C	40°C	50°C	0 m*M*	300 m*M*	450 m*M*
Maximum water solubility (g/L)	1.52	1.75	2	1.72	1.95	2.2	1.51	2.11	2.9

From refs. 5, 15, 17, and 26.

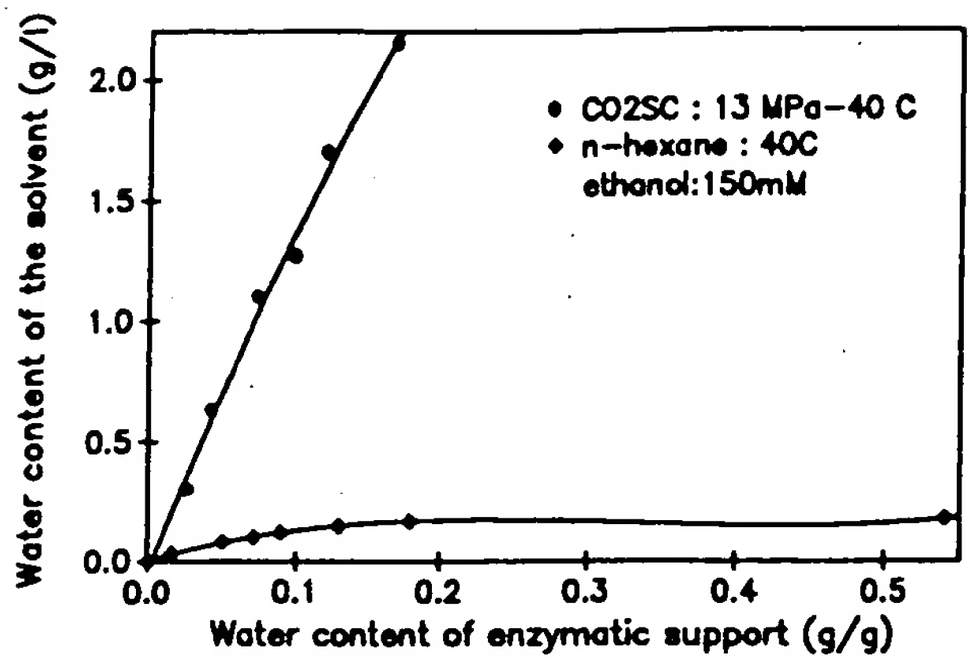


Figure 6. Adsorption isotherm comparison in $SCCO_2$ (13 MPa, 40°C, ethanol: 150 m*M*) and *n*-hexane (40°C, ethanol: 150 m*M*).

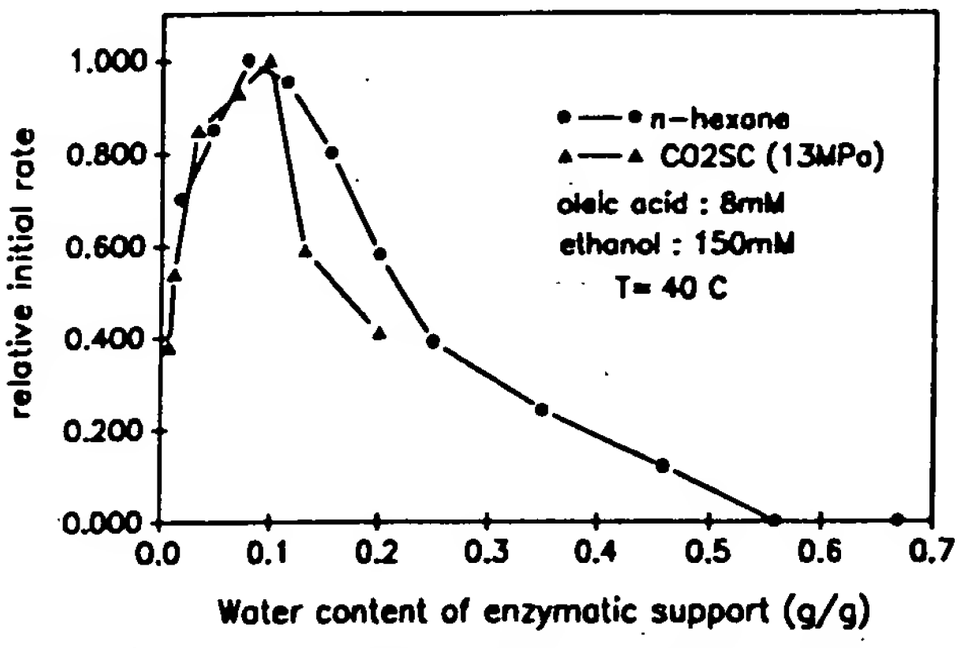


Figure 7. Influence of water content of enzymatic support on enzyme activity in $SCCO_2$ (13 MPa, 40°C) and *n*-hexane (40°C).

When considering the decrease of the initial velocity, presented in Figure 8, with increasing ethanol concentration, it may first be deduced that this two-substrate reaction is inhibited by ethanol excess.

Nevertheless, the decrease of initial velocity may also be attributed to an entrainer effect by ethanol that "dries" the support and lowers the activity (left side of the bell-shaped curve 7), but experiments with a 15% water content of the solid (right side of the bell-shaped curve 7) present the same (Fig. 9) decreasing activity with increasing ethanol concentration, which would contradict this hypothesis. The mechanistic inhibition is therefore validated. No inhibitions effect by oleic acid or ethyloleate has been detected.

Elsewhere[6] the same reaction has been tested in *n*-hexane. A Ping-Pong Bi-Bi mechanism with inhibition by the ethanol substrate has been suggested because of a very good correlation between experimental results and kinetic curves given by its scheme, even if mechanism determination cannot rest on kinetic data alone. This mechanism is easily described in a reciprocal coordinate representation where parallel lines are obtained for low ethanol concentrations. The slopes of these lines are increasing with higher ethanol concentrations and the intercepts of these lines tend to a limiting value equal to $1/V_{max}$.

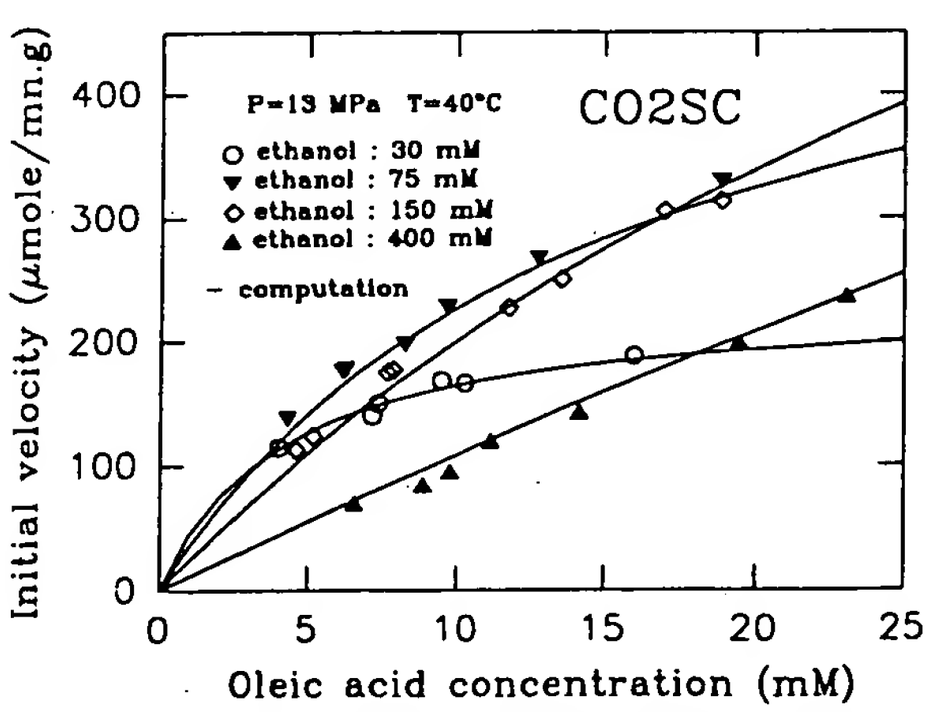
Experimental values obtained in $SCCO_2$ (Fig. 8) tend to prove that the mechanism is similar in both media.

The reaction scheme is described in Fig. 10. Oleic acid is linked to the enzyme, leading to a substrate–enzyme complex which, as an intermediary, delivers water. This is followed by the linkage of ethanol into a second complex that delivers the product and ensures enzyme recovery. Another parallel pathway is the direct linkage of ethanol to the enzyme leading to an inactive complex.

The equation of the model from Segel[22] is written as

$$\frac{V}{V_{max}} = \frac{[Ol][Eth]}{K_{m(ol)}[Eth](1 + [Eth]/K_i) + K_{m(eth)}[Ol] + [Ol][Eth]}$$

Graphical determination of the involved parameters may be carried out because the limiting value of the intercept is equal to $1/V_{max}$ and the plot of the increasing slopes against ethanol concentration will provide the inhibition constant $K_{i(eth)}$ of ethanol and affinity



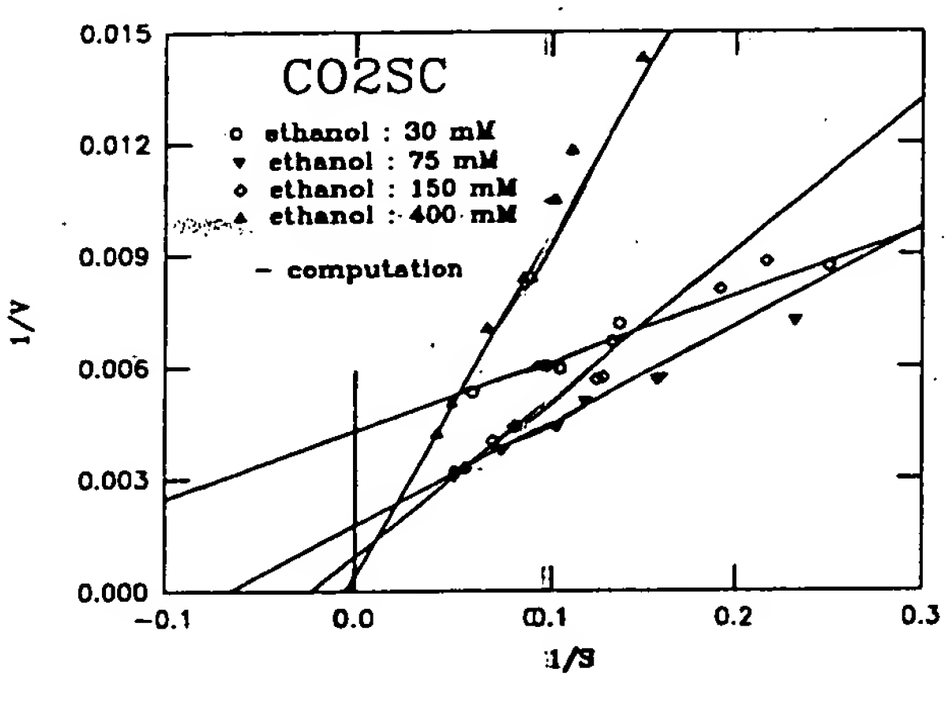


Figure 8. Enzymatic activity in $SCCO_2$ (13 MPa, 40°C) vs. oleic acid and ethanol concentration and its reciprocal coordinate representation.

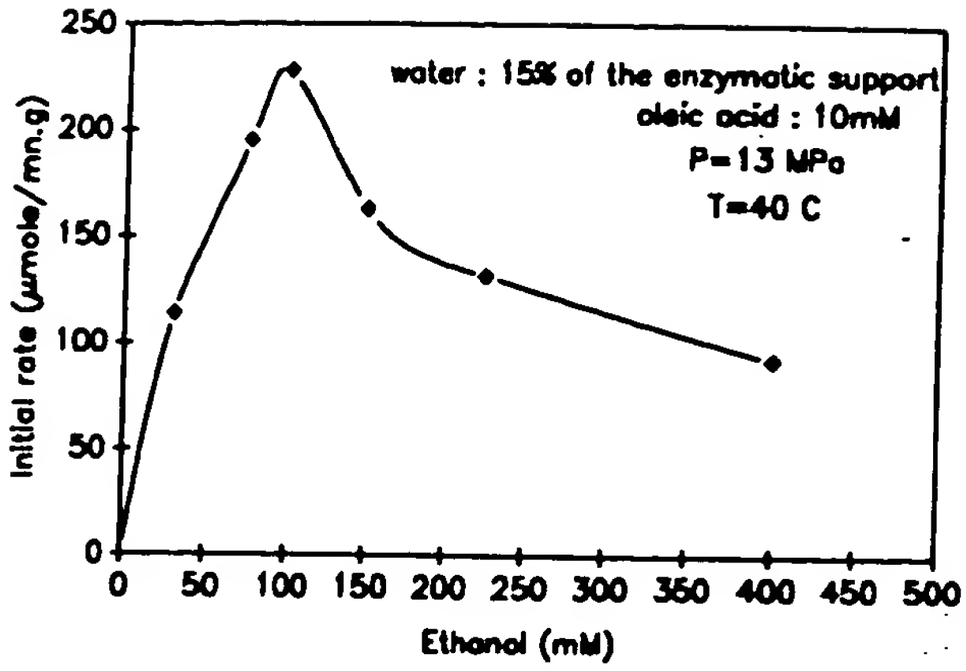


Figure 9. Influence of ethanol concentration on enzyme activity at water content above optimum concentration.

constant $K_{m(ol)}$ of ethanol for the immobilized enzyme. Because graphical determination appears rather inaccurate, we have performed direct parametric identification on the model equation (Gauss–Newton algorithm). On curve 8, symbols represent experimental values whereas lines represent the kinetic model. Apparent kinetic parameters, with an average relative error of 5.3% and a maximum error of 15.7% between experimental and calculated values, are presented in Table II.

In the case of an immobilized catalyst the determination of a mechanism is always delicate because of interaction of mass transfer phenomena (internal and external diffusion). In our apparatus, agitation is a combination of magnetic stirring and jet mixing due to the recirculation loop. The external diffusion has been tested by performing experiments without magnetic stirring and with variable rotating speeds of the pump. No difference in reaction rates has been detected. We may assert that due to excellent diffusivities in supercritical CO_2, external mass transfer has no influence. In the case of internal mass transfer, experiments with different sizes or porosities of support would have given necessary information, but the commercial catalyst used here was not available in different patterns. A predictive calculus (estimation of Thiele modulus) was judged too hazardous because of the lack of suitable data. Nevertheless, these internal mass transfer limitations may be assumed negligible without great error due to the very good transport properties of supercritical CO_2. Erickson et al.,[8] in the study of transesterification of trilaurin with palmitic acid in $SCCO_2$, have estimated a Damkhöller number of about 10^{-5} (ratio of the characteristic reaction rate to the characteristic external diffusion rate) and a Thiele modulus about 10^{-2}. These values lead to estimation of internal and external effectiveness factors approaching unity, and so these authors concluded in favor of the noninfluence of mass transfer phenomena.

COMPARISON OF RESULTS WITH ORGANIC SOLVENT

Kinetic constants calculated in *n*-hexane previously[6] cannot be used for a comparison because they correspond to 5% water content of the solid which is not the optimum. Kinetic curves at the optimum water content (10%) have been redetermined in this article and are shown in Figure 11.

Corresponding kinetic parameters have been calculated by the same parametric identification method and values are given in Table II.

The apparent maximum velocity appears to be twice as high in *n*-hexane. A possible explanation may be drawn from the influence of the pH of aqueous phase surrounding the enzyme. Indeed, CO_2 can diffuse in this pseudoaqueous phase, trapped within the enzymatic support, and may acidify the medium. Consequently, the enzyme would be in less favorable conditions for the reaction.

Such effects have been supposed in lipase-catalyzed hydrolysis, esterification, or interesterification by Cambou and Klibanov in 1984[3] and Abraham et al. in 1988.[1] For instance, in ester hydrolysis, products of the reaction such as polar acids are supposed to dissolve in nonnegligible amounts in water and to acidify the pH in the microenvironment of the catalyst, leading to a loss of activity. Recently, Valinety et al.[25] suggested a method for measuring the pH of an aqueous phase trapped within biocatalyst particles. They used very hydrophobic esters of fluorescein that remain entirely in the organic phase and are ionized in relation with the pH of the aqueous phase. They have demonstrated that during hydrolysis of dodecyl acetate liberation of acetic acid by Liposyme leads to the decrease of the pH. In our case, this method, adapted to supercritical solvents, could be a way of investigating this hypothesis.

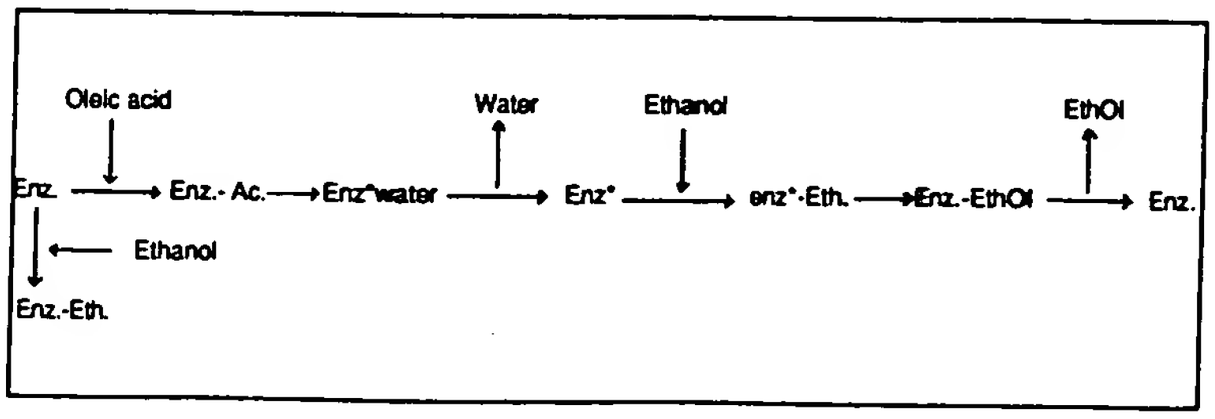


Figure 10. Schematic representation of the Ping-Pong Bi-Bi with inhibition by ethanol mechanism.

Table II. Kinetic parameters obtained in $SCCO_2$ (13 MPa, 40°C) and *n*-hexane at 40°C.

	V_m (mmol/min · g)	$K_{m(ol)}$ (mM)	$K_{m(eth)}$ (mM)	K_i (mM)
$SCCO_2$	14	170	1600	65
n-Hexane	23	450	600	60

Average relative error between modelized and experimental values: 5.3% and 4.2% with a maximum about 15.7% and 14.3% in $SCCO_2$ and in *n*-hexane, respectively.

Considering the apparent affinity constant of enzyme for oleic acid ($K_{(ol)}$), it has been found that the affinity of oleic acid is greater in $SCCO_2$, resulting from a probably lower diffusional limitation in the case of the supercritical solvent, but surprising as it may seem, the contrary is observed for the apparent affinity constant for ethanol ($K_{(eth)}$).

This could also be attributed to the immobilization of the enzyme that may induce, as we have already observed for water, a different partition for oleic acid and ethanol between enzymatic support and the two solvents. Indeed, oleic acid is more soluble in *n*-hexane than in $SCCO_2$ (in the range of studied pressures), leading to a concentration near the protein greater in $SCCO_2$. This effect, added to the diffusional effect, could explain the better apparent affinity constant of oleic acid in the case of $SCCO_2$.

On the contrary, in the case of *n*-hexane, ethanol is distributed favorably on the solid phase. Consequently, ethanol concentration is more important in the vicinity of the enzyme in hexane and leads to a less favorable apparent affinity constant in the case of $SCCO_2$ despite the benefit of the decreased diffusional limitation.

We are currently working on ways to verify these hypotheses in order to improve control of the parameters which may influence performances of enzymatic reaction in supercritical carbon dioxide.

Nevertheless, experimental initial velocities, for oleic acid concentration lower than 25 mM (this limiting concentration arises from the restricted solubility of oleic acid in $SCCO_2$) are very similar in both media. In $SCCO_2$, they are only 10% weaker than in *n*-hexane.

CONCLUSION

In this study, Lyposyme from *M. miehei* appears quite stable in supercritical carbon dioxide in a range of pressure from 13 to 18 MPa. Stability is similar in $SCCO_2$ and in *n*-hexane. These results, obtained over a period of 6 days, lead to the possibility of using $SCCO_2$ in a continuous enzymatic reactor. Finally, water has proved to be an important denaturing factor.

The main interest of this work lies in the fact that it is the first complete study of the kinetic behavior of enzymatic reaction in $SCCO_2$ in comparison with a conventional organic solvent, *n*-hexane. This study is based on the determination of kinetic constants of reaction related to a mechanism, suggested by a very good correlation of calculated kinetics curves with experimental data. The results provide an accurate comparison of the kinetics in the two solvents because optimum water content of the solid phase, which has a dramatic influence, has been achieved in each case.

It may be concluded that $SCCO_2$ seems to be an appropriate solvent, very similar to an organic solvent from the point of view of stability and kinetics of enzyme, provided that special attention is paid to the water content of the enzymatic support.

As a prospective conclusion, our objective would be to demonstrate potentialities of supercritical fluids in actual reactor operation and in postreactional processing. Indeed, in our experiments, performed in a well-stirred enzyme reactor, external diffusion was considered to play a negligible role, but in a continuous fixed bed of immobilized enzyme, this phenomenon could be critical. Recently, Lim et al.[13] proved that mass transfer rates in a fixed bed are much greater in supercritical conditions than for standard liquid–solid or gas–solid systems. This improvement is the consequence of important mass transfer enhancement by natural convection effects in supercritical conditions.

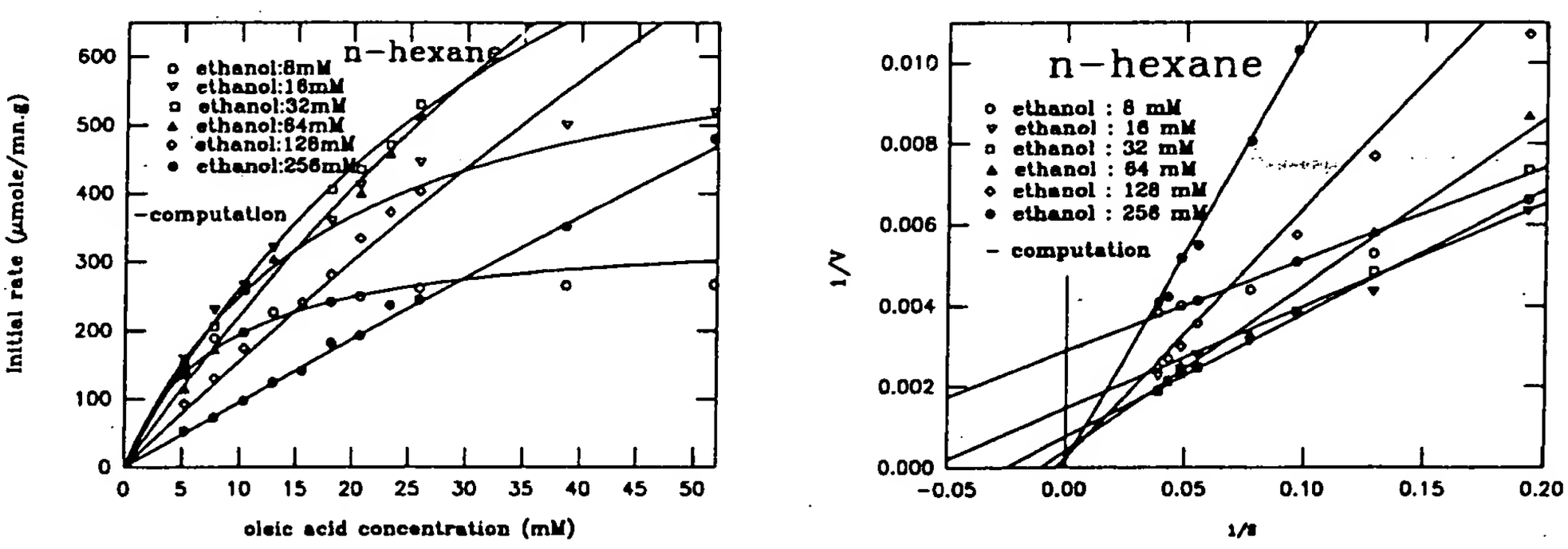


Figure 11. Enzymatic activity in *n*-hexane (40°C) vs. oleic acid and ethanol concentration and its reciprocal coordinate representation.

Moreover, an advantage of supercritical fluids, considering a complete process, is that after reaction and depressurization a mixture of products and nonreacted substrates may be obtained without traces of solvent. The natural follow-up is to connect, after the reactor, separators using depressurization stages to desolubilize compounds issued from the reaction one after the other.

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Enzymatic Esterification of Fatty Acid Mixtures from M Anhydrous Milk Fat with Canola Oil in Supercritical Ca

Zer-Ran Yu,*,† Syed S. H. Rizvi,† and John A. Zollweg‡

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The ester ethyl oleate was synthesized from oleic acid and ethanol by using *Candida cylindracea*, immobilized on Celite 545 and in supercritical carb (SC-CO_2) as the solvent. The enzymatic esterification reaction is much f CO_2 than in organic solvents. This technique was then applied to the hydroly of milk fat to determine the feasibility of using it as a process to enhance chromatographic (GC) analysis of the ethyl esters showed that short-chain (C_4–C_8) esterify more completely than long-chain fatty acids (C_{16}–C_{18}). Th was then applied to interesterify a mixture of triglycerides from canola oil anhydrous milk fat (AMF). The starting mixture contained predominan triglycerides from the CNO and C_{32}–C_{38} triglycerides from the AMF. T exhibited predominantly the C_{42}–C_{50} and C_{54} triglycerides.

Introduction

Lipase (glycerol ester hydrolase) comprises a group of enzymes that catalyze the hydrolysis of triacylglycerols to give free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. Since this reaction is reversible, lipase can also catalyze (i) the formation of esters from alcohols and fatty acids and acylglycerols from glycerol and fatty acids (esterification) and (ii) the exchange between free fatty acids in fats and oils (acidolysis) and the ester exchange between two fats and oils (interesterification). The term transesterification includes both the reactions of acidolysis and of interesterification (Yamane, 1987).

Esterification can be used to change the physical and functional properties of edible fats and oils. The process of esterification can be accelerated either by chemical catalysts such as sodium metal, sodium–potassium alloy, and sodium alkoxide or by a biocatalyst such as lipase. Enzymatic esterification is of great interest since it offers several advantages over chemical methods, namely, selective and positionally specific fatty acid exchange, milder reaction conditions, and tolerance of water and free fatty acids (Macrae, 1983a; Sreenivasan, 1978).

It is possible to use enzymatic esterification to produce specific fatty acid esters similar to the flavorants used in margarine, imitation dairy products, confections, and other prepared foods (Nelson, 1972). Formation of flavor esters can be catalyzed by immobilized *Candida cylindracea* lipase in organic solvents (Marlot et al., 1985; Gillies et al., 1987; Langrand et al., 1988). This lipase exhibits activity over a wide pH range (2–8.5) (Macrae, 1983b), has been shown to esterify ethanol with the fatty acids found in butter triglycerides (Kanisawa, 1983), and moreover, preferentially esterifies short-chain fatty acids (C_4–C_8) (Kanisawa, 1983; Gillies et al., 1987). Another common and effective lipase from *Mucor miehei* can also be used to synthesize esters from numerous alcoholic and acidic substrates (Marlot et al., 1985; Langrand et al., 1988; Omar et al., 1989).

For the manufacture of high-value confectionery fats, e.g., those low in caloric value and high in unsaturation, transesterification has been us and functional properties of f fication exchanges fatty acids fr of another in the presence o example, commercially availabl was used to interesterify canola o et al., 1988). Coleman and Ma interesterification of triglyceri and olive oils by using *Candi* catalyst. Wisdom et al. (1985, 19 interesterification of fats using *Rhizopus arrhizus* and *Asperg*

Supercritical carbon dioxid solvent, especially for food and tions. Compared to liquid solv inexpensive, is nontoxic, and p high mass transfer and diffusio suited for enzymatic reaction dioxide has been used, for ex reactions catalyzed by polypher al., 1985) and cholesterol oxidas Chi et al. (1988) studied lipa interesterification in SC-CO_2. successfully used SC-CO_2 to tra and nonyl acetate from ethyl ac ing alcohol. Marty et al. (1990) esterification with ethanol by us from *Mucor miehei* in both S solvents. They compared the e two solvents with respect to the c water content, and the enzyme mont and his co-workers (1991) u from *Mucor miehei* to produ myristic acid and ethanol under

The objective of this investiga the immobilized *Candida cylin* to produce ethyl oleate in the SC-CO_2 as the solvent. The pressure and temperature, react content of the immobilized lip product, and enzyme stabilit conditions were then applied to t from the fatty acids of milk fat.

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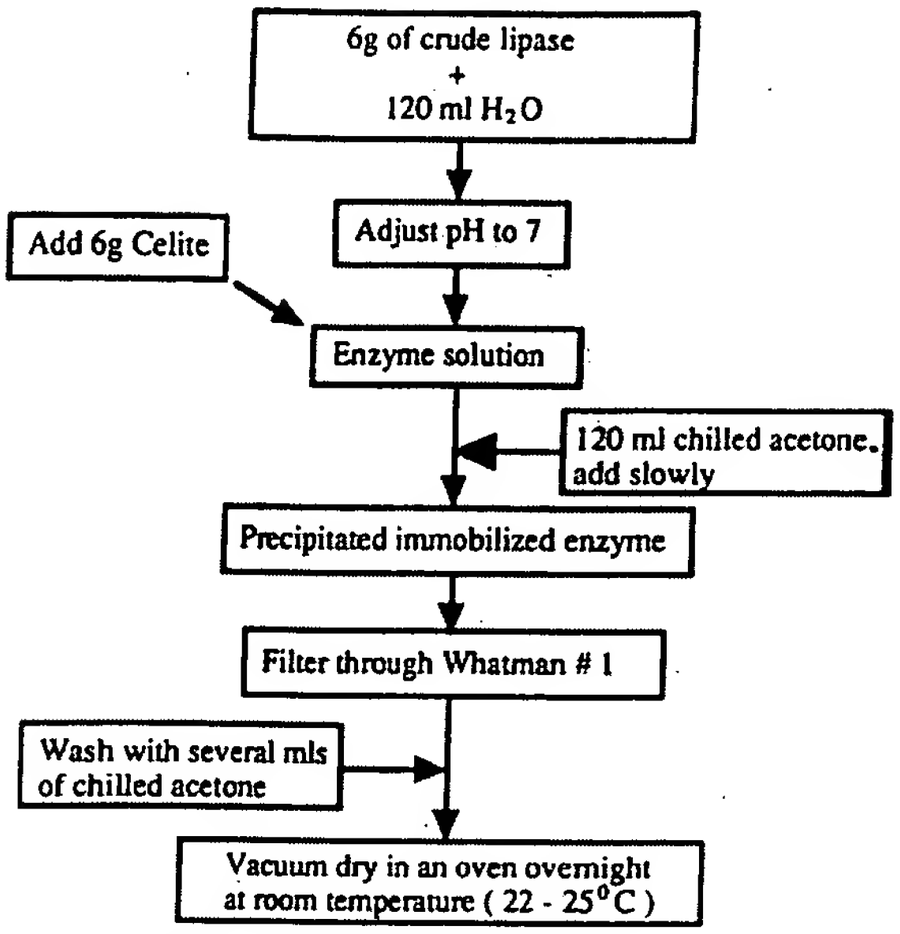

Figure 1. Procedure for immobilized lipase preparation.

terification of anhydrous milk fat with canola oil in SC-CO_2 was also studied using the same immobilized lipase.

Materials and Methods

Materials. The lipase *Candida cylindracea* for immobilization studies was obtained from Sigma Chemical Co. (type VII, EC 3.1.1.3). It contains 4570 units/mg of protein of lipolytic activity as defined by the supplier. Celite 545, used for immobilization, was purchased from Fisher Scientific Co. The oleic acid and 1-octanol (99% pure) were from Sigma. Absolute ethanol was from Quantum Chemical Co. Anhydrous milk fat (AMF) was prepared by melting butter fat and refining with centrifugation and filtration. It had 98% oil and 0.1% water content, confirmed by the method of AOCS (1989). The fatty acids of butter fat were prepared by hydrolysis of AMF with 0.1 N NaOH in ethanol at 75 °C for 30 min, followed by neutralization with 0.07 N HCl. The canola oil (Puritan) was commercially available from Procter & Gamble Food Ltd. Carbon dioxide (99.999% pure) was purchased from Airco Inc.

Enzyme Immobilization. The immobilized enzyme was prepared according to the method outlined in Figure 1 and then stored at 0–5 °C. Before use, the immobilized enzyme was hydrated by adding 1 mL of water to 1 g of immobilized lipase.

Solubility Measurement. The solubility of oleic acid in the oleic acid + ethanol + SC-CO_2 system and the solubility of the AMF + CNO mixture in SC-CO_2 were studied following the experimental design and procedures of Yu et al. (1992). The solubilities of oleic acid were obtained from different ratios of ethanol to oleic acid (100% oleic acid, 5% ethanol in oleic acid, 10% ethanol in oleic acid, and 20% ethanol in oleic acid) at 40 °C and pressures between 10 and 30 MPa. The solubility of a 1:1 mixture of AMF and CNO in SC-CO_2 was measured at 40 °C and pressures between 10 and 30 MPa.

The concentration of ethyl oleate in the fluid phase was measured by the method of gas chromatography (details in the analytical section). The amount of CO_2 was determined by expanding the samples into a vessel, measuring the temperature and pressure, and using published density data for pure CO_2 at these conditions (Angus et al., 1976). The concentration of ethanol was

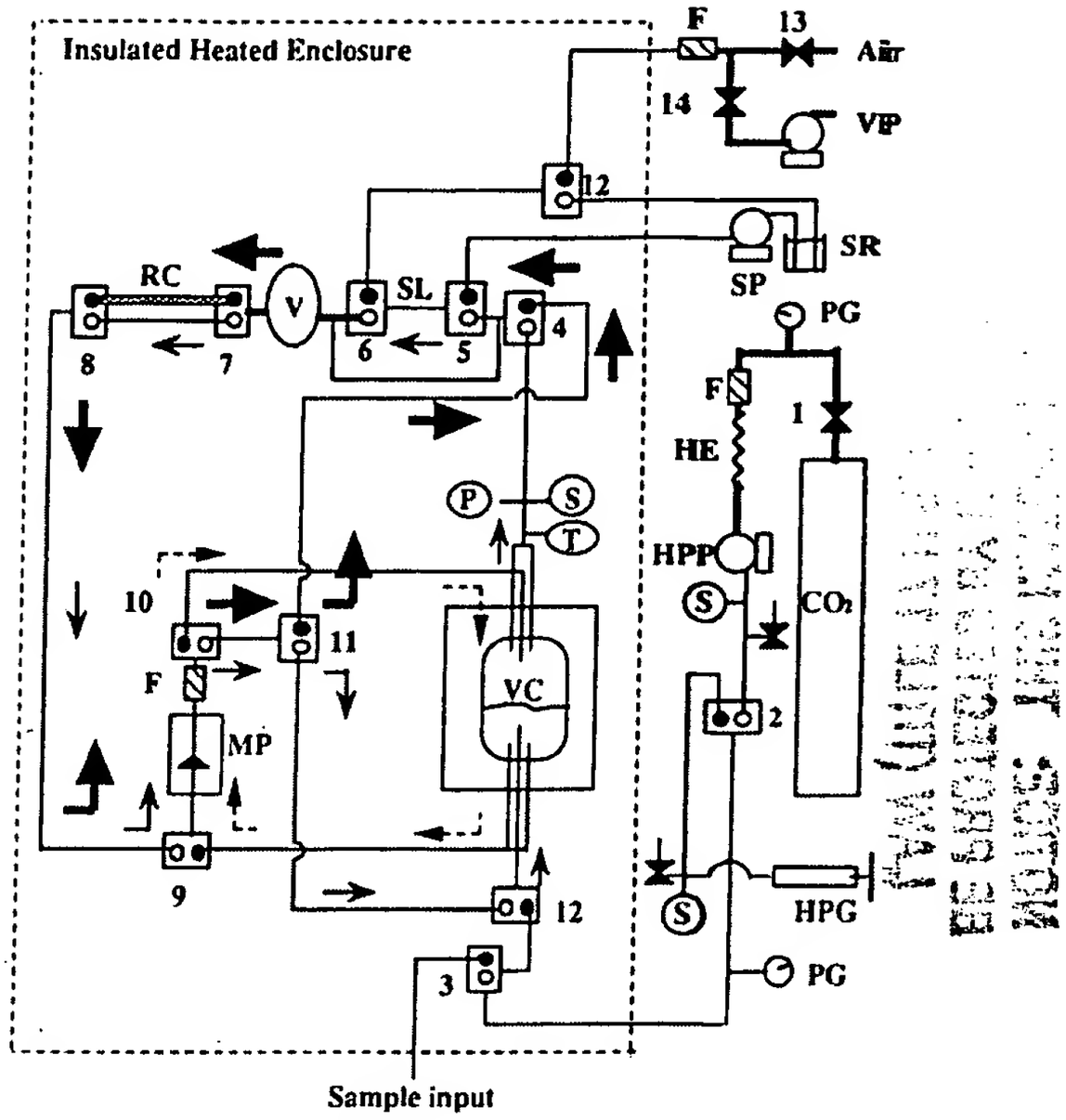

Figure 2. Schematic diagram of supercritical carbon dioxide system for enzymatic esterification: [O ●], three-way/two-stem combination valves (O front side, ● back side); ɪ, two-way straight valve; —, 316 stainless steel tubing, 3.18 mm o.d. × 1.40 mm i.d.; —, 316 stainless steel tubing, 6.35 mm o.d. × 4.57 mm i.d.; HPP, high-pressure pump; MP, magnetic pump; VP, vacuum pump; SP, solvent pump; HPG, high-pressure generator; HE, heat exchanger; *T*, temperature of system; *P*, pressure of system; PG, pressure gauge; S, safety valve; F, filter; VC, view cell; SL, sample loop; V, fluid phase reservoir; SR, solvent reservoir; RC, reactor column.

not measured because of the design limitations of the equipment. The solubility of the AMF + CNO mixture in SC-CO_2 was determined by the gravimetric method.

Esterification under Supercritical Conditions. There are two steps to perform in the enzymatic reactions of this study: (i) substrate extraction and (ii) substrate reaction.

Figure 2 shows a schematic diagram of the apparatus. Prior to starting the experiment, the system was flushed several times with *n*-hexane. Sequentially, the system was dried with air and evacuated using a vacuum pump (VP). The head of the high-pressure pump (HPP) was cooled to −10 °C. One of the substrates (10 wt % ethanol in oleic acid, 10 wt % ethanol in fatty acid mixtures from milk fat, or a 1:1 (wt) mixture of AMF and CNO) was fed into the system and then extracted with SC-CO_2 as solvent. A sample of 35 g was introduced through valves 3 and 12 into the view cell (VC) as shown in Figure 2. The high-pressure pump (HPP) was used to pump carbon dioxide into the view cell (VC). The pressure level was adjusted using a high-pressure generator (HPG) as a variable volume for the system. The magnetic pump (MP) circulated the fluid or liquid phase as indicated by solid or dashed arrows, respectively. The extract in the fluid phase was pumped in the loop using valves 5–8 but bypassing the reactor column (RC). This was done until phase equilibrium was established. The equilibrium times required for extracting the fatty acid mixtures and triglyceride mixtures were experimentally determined to be 4 and 8 h, respectively.

When the extraction was done, the extract was intro-

Enzymatic Esterification of Fatty Acid Mixtures from Milk Fat and Anhydrous Milk Fat with Canola Oil in Supercritical Carbon Dioxide

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The ester ethyl oleate was synthesized from oleic acid and ethanol by using the lipase, *Candida cylindracea*, immobilized on Celite 545 and in supercritical carbon dioxide (SC-CO_2) as the solvent. The enzymatic esterification reaction is much faster in SC-CO_2 than in organic solvents. This technique was then applied to the hydrolysis products of milk fat to determine the feasibility of using it as a process to enhance flavor. Gas chromatographic (GC) analysis of the ethyl esters showed that short-chain fatty acids (C_4–C_8) esterify more completely than long-chain fatty acids (C_{16}–C_{18}). The technique was then applied to interesterify a mixture of triglycerides from canola oil (CNO) and anhydrous milk fat (AMF). The starting mixture contained predominantly C_{52}–C_{56} triglycerides from the CNO and C_{32}–C_{38} triglycerides from the AMF. The product exhibited predominantly the C_{42}–C_{50} and C_{54} triglycerides.

Introduction

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For the manufacture of high-value confectionery fats, e.g., those low in caloric value and high in unsaturation, transesterification has been used to modify the physical and functional properties of fats and oils. Transesterification exchanges fatty acids from one fat or oil with those of another in the presence of lipase as catalyst. For example, commercially available porcine pancreatic lipase was used to interesterify canola oil with lauric acid (Thomas et al., 1988). Coleman and Macrae (1980) demonstrated interesterification of triglycerides in a mixture of coconut and olive oils by using *Candida cylindracea* lipase as catalyst. Wisdom et al. (1985, 1987) also showed enzymatic interesterification of fats using immobilized lipases from *Rhizopus arrhizus* and *Aspergillus* sp.

Supercritical carbon dioxide (SC-CO_2) is a unique solvent, especially for food and pharmaceutical applications. Compared to liquid solvents, SC-CO_2 is relatively inexpensive, is nontoxic, and possesses low viscosity and high mass transfer and diffusion rates—properties well-suited for enzymatic reactions. Supercritical carbon dioxide has been used, for example, as a medium for reactions catalyzed by polyphenol oxidase (Hammond et al., 1985) and cholesterol oxidase (Randolph et al., 1988). Chi et al. (1988) studied lipase-catalyzed triglyceride interesterification in SC-CO_2. Van Eijs et al. (1988) successfully used SC-CO_2 to transesterify isoamyl acetate and nonyl acetate from ethyl acetate with the corresponding alcohol. Marty et al. (1990) demonstrated oleic acid esterification with ethanol by using an immobilized lipase from *Mucor miehei* in both SC-CO_2 and *n*-hexane as solvents. They compared the enzymatic reactions in the two solvents with respect to the concentration of reactants, water content, and the enzyme stability. Recently, Dumont and his co-workers (1991) used an immobilized lipase from *Mucor miehei* to produce ethyl myristate from myristic acid and ethanol under supercritical conditions.

The objective of this investigation was to establish that the immobilized *Candida cylindracea* lipase can be used to produce ethyl oleate in the presence of ethanol with SC-CO_2 as the solvent. The optimum conditions of pressure and temperature, reactant concentrations, water content of the immobilized lipase, water content of the product, and enzyme stability were studied. These conditions were then applied to the synthesis of ethyl esters from the fatty acids of milk fat. The enzymatic interes-

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duced into the reactor column (RC) where enzymatic esterification occurred. This was done by closing the front side of valves 4–8 and 11 and opening the back side of valves of 4, 7, 8, and 11, as indicated by the large solid arrows in Figure 2. The reactor column (RC) was a 250 mm long × 6.35 mm o.d. (4.57 mm i.d.) 316 stainless steel tube packed with immobilized enzyme and sealed with 7-μm filters (Supelco Inc.) at both ends. After a fixed reaction time, the products in the sample loop (SL) were transferred to the solvent reservoir (SR). The pressure in SL was first gradually reduced to atmospheric pressure through the SR. The trapped sample in SL was then rinsed three times with hexane (30 mL/rinse) using a solvent pump (SP). The combined washings were analyzed by gas–liquid chromatography. A 75 cm^3 vessel (V) was installed after valve 6 to reduce pressure loss during sampling. The pressure change in the system was about 0.05 MPa per sampling. Five samples were collected in each experiment.

Analytical. The ethyl oleate, ethyl ester mixtures of AMF, and triglycerides of anhydrous milk fat with canola oil were directly analyzed on a gas–liquid chromatograph (GC) equipped with a flame ionization detector (FID) (Hewlett-Parkard, 5890) using helium (1.5 mL/min) as the carrier gas. The ethyl oleate was directly analyzed on a capillary column coated with OV-225 (DB-225, 30 m × 0.25 mm i.d.; 0.25-μm film, J & W Scientific Inc.) at 200 °C for 10 min. The same capillary column was used for the analysis of ethyl ester mixtures of AMF. The column was held at 60 °C for 2 min, followed by a rise of 4 °C/min to 220 °C, and finally held for 10 min at 220 °C. The triglycerides of anhydrous milk fat with canola oil were directly analyzed in a capillary column coated with SE-30 (25 m × 0.25 mm i.d.; 0.1-μm film, Chrompack Co.) at 280 °C for 10 min, followed by 1 °C/min to 330 °C, and finally held for 10 min at 330 °C. The analysis of each sample was performed in duplicate. The standard error in the analysis was calculated to be 5% at most.

Results and Discussion

Esterification of Ethanol + Oleic Acid in SC-CO_2. (*1*) ***Effects of the Reaction Conditions and Substrate Concentration.*** Since the optimal activity of *Candida cylindracea* lipase occurs in the temperature range 30–40 °C, the enzymatic esterification studies were conducted at 40 °C. The pressure of 13.6 MPa was determined by the solubility of oleic acid and ethanol in SC-CO_2. According to a study by Marty et al. (1990), the concentrations of about 20 mmol/L oleic acid and 150 mmol/L ethanol are the best choice for synthesis of ethyl oleate. The solubilities of oleic acid obtained from different ratios of ethanol to oleic acid at 40 °C and pressures between 10 and 30 MPa are shown in Figure 3. The solubilities of oleic acid are about 20 and 25 mmol/L, respectively, from 0 and 10 wt % ethanol in oleic acid at 13.6 MPa and 40 °C.

Previous research (Suzuki et al., 1990) has shown that SC-CO_2 at 40 °C becomes saturated with ethanol at 6 g/L (130 mmol/L) when the pressure is 8.2 MPa. Therefore, the solubility of ethanol in SC-CO_2 could be between 20 and 130 mmol/L when the pressure is greater than 8.2 MPa and the liquid phase is a mixture containing ethanol. Since ethanol is a more volatile compound than oleic acid, the solubility of ethanol in SC-CO_2 from a liquid phase containing 10% ethanol + 90% oleic acid should be similar to that of 100% ethanol. Also, ethanol exhibits a low critical pressure at 8.2 MPa and 40 °C for the mixture of pure ethanol in SC-CO_2 (Suzuki et al., 1990). Therefore,

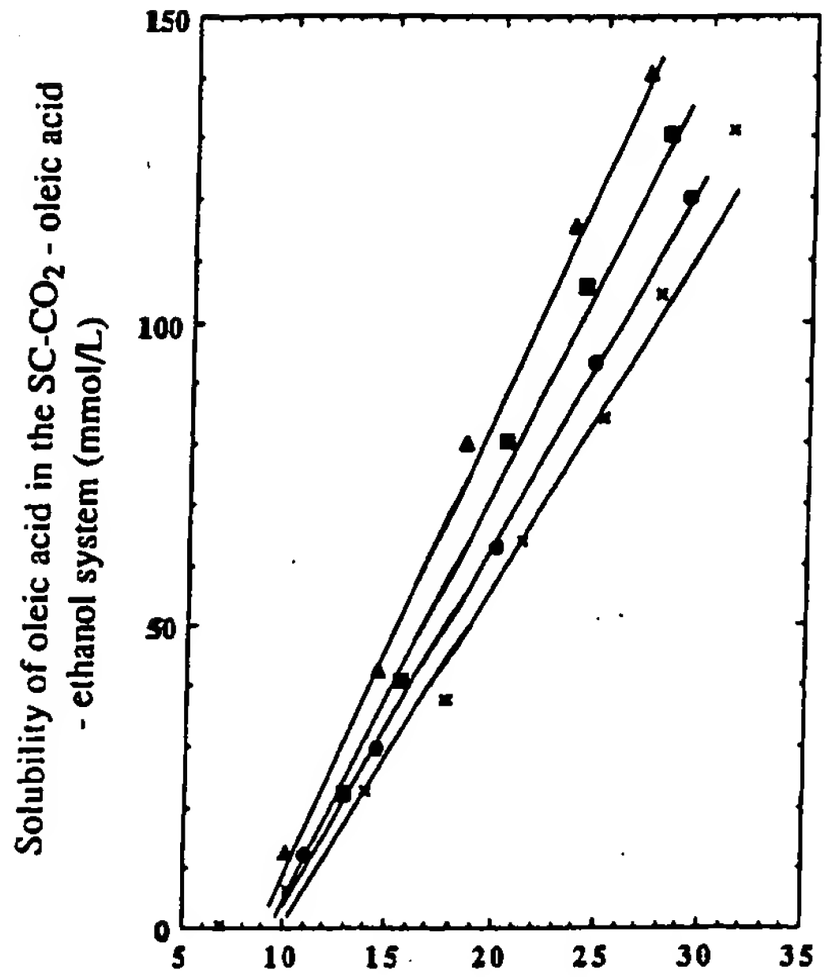

Figure 3. Solubilities of oleic acid in SC-CO_2 in various ethanol concentrations at 40 °C: ×, 0 wt %; ●, 5 wt %; ■, 10 wt %; ▲, 20 wt %.

those conditions were selected for the synthesis of ethyl oleate in SC-CO_2.

(*2*) ***Effect of Water Content.*** The enzymatic esterification is not only affected by the solubilities of reactants in SC-CO_2 and operation conditions but is also affected by other factors, including the amount of water in the immobilized lipase, water content of the product, and lipase activity and stability. It is known that lipases need a small amount of water to maintain their active conformation (Zaks and Klibanov, 1988). However, excess water in the immobilized lipase and in the product has negative effects (hydrolysis reaction) on the ester synthesis.

In this study, the conversion of ethyl oleate was measured at different water contents in the immobilized lipase after a 1-h reaction time. These results as shown in Figure 4 indicate that the 30% production of ethyl oleate in SC-CO_2 occurs when 1 mL of water is added to 1 g of immobilized *Candida cylindracea* lipase. However, other studies have shown widely varying optimum water concentrations for lipase-catalyzed esterifications in SC-CO_2 (Chi et al., 1988; Marty et al., 1990, 1992). Marty's two experiments, which were performed using the *Mucor miehei* lipase supported on macroporous anionic resin beads at identical conditions, gave very different results. If we assume that Marty's data published in 1992 are correct, the fact that our water concentration is much larger than his (0.1 mL of water is added to 1 g of immobilized lipase) can be explained due to water adsorption on the hydrophilic support.

When the immobilized lipase contains excess water, the ethyl oleate can be hydrolyzed to ethanol and oleic acid. Even though there is a low water concentration in this system (2 mL of water in 100 mL of SC-CO_2), there is still the potential for the hydrolysis reaction. The reaction was studied to determine whether the hydrolysis of methyl oleate could occur using the same concentrations of immobilized lipase and water and operating at the same pressure and temperature as in the esterification reaction. The experimental result showed that there was a 10% hydrolysis reaction after 30 min of reaction.

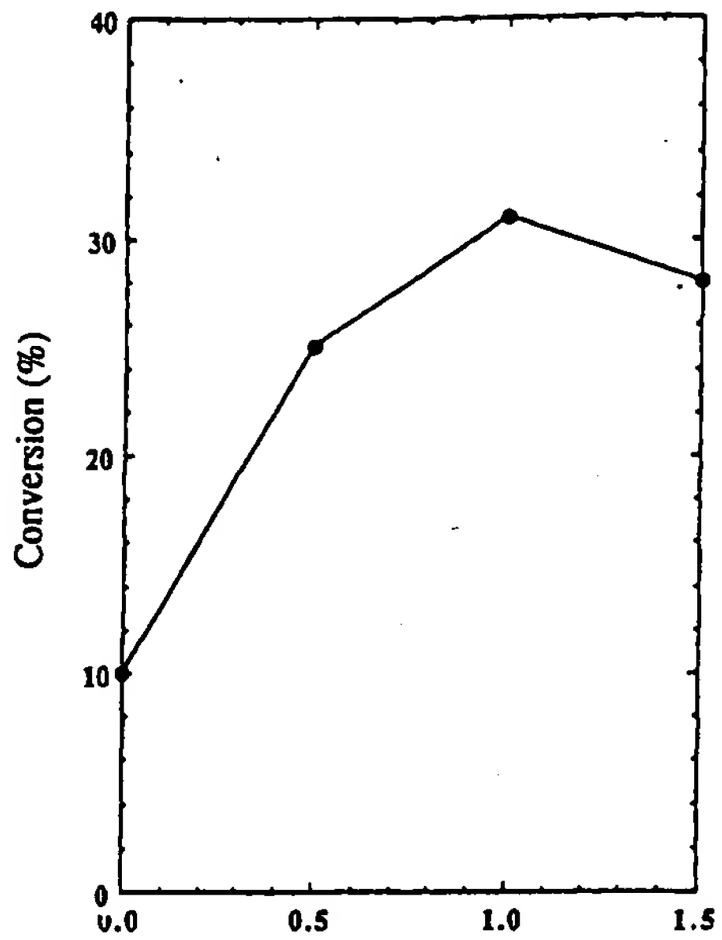

Figure 4. Conversion of ethyl oleate versus water content in the immobilized lipase.

Since a preliminary experiment indicated that accumulation of water slowed down the reaction, molecular sieves were used to remove water produced during the reaction. The molecular sieves were packed in a 50-mm length of 6.35 mm o.d. (4.57 mm i.d.) 316 stainless steel tubing capped with 7-μm filters at both ends. The column containing the molecular sieves was installed after the reaction column (RC). Circulation of the reactant medium through the molecular sieves removed sufficient water from the product.

(*3*) ***Enzymatic Activity.*** The degree of conversion of esterification was determined by measuring both the free fatty acid and esterified fatty acid concentrations. About 25 mmol/L oleic acid was reacted using 2 g of immobilized lipase in a 100-mL SC-CO_2 system. The reactants were circulated through the lipase column at a flow rate of 40 mL/min. The synthesis of ethyl oleate using the *Candida cylindracea* lipase resulted in about 30% conversion. Figure 5 compares the percent conversions of ethyl oleate, 30%, and octyl oleate, 75%, to the reported value of 95% for ethyl butyrate (Gillies et al., 1987). In Gillies' experiment, the reaction was performed using 5 g of immobilized lipase in 100 mL of heptane containing 250 mmol/L butyric acid and 400 mmol/L ethanol. These results implied that the lipase exhibited enzymatic activity to different substrates. Moreover, the enzymatic esterification in SC-CO_2 had a higher initial velocity and a shorter reaction time.

According to the studies of Chi et al. (1988) and Marty et al. (1990), the choice of solvent, either SC-CO_2 or *n*-hexane, does not affect the percent conversion when using the same reactants. In this study, the percent conversions of ethyl oleate and octyl oleate obtained using SC-CO_2 are relatively close to the reported values (Lazar et al., 1986) of 27% for ethyl oleate and 79% for octyl oleate in *n*-hexane. Hence, one could predict a value of 95% conversion for the synthesis of ethyl butyrate using *Candida cylindracea* lipase in SC-CO_2.

(*4*) ***Enzymatic Stability.*** Figure 6 compares the stability of *Candida cylindracea* lipase supported on Celite 545 (this work) and *Mucor miehei* lipase supported on

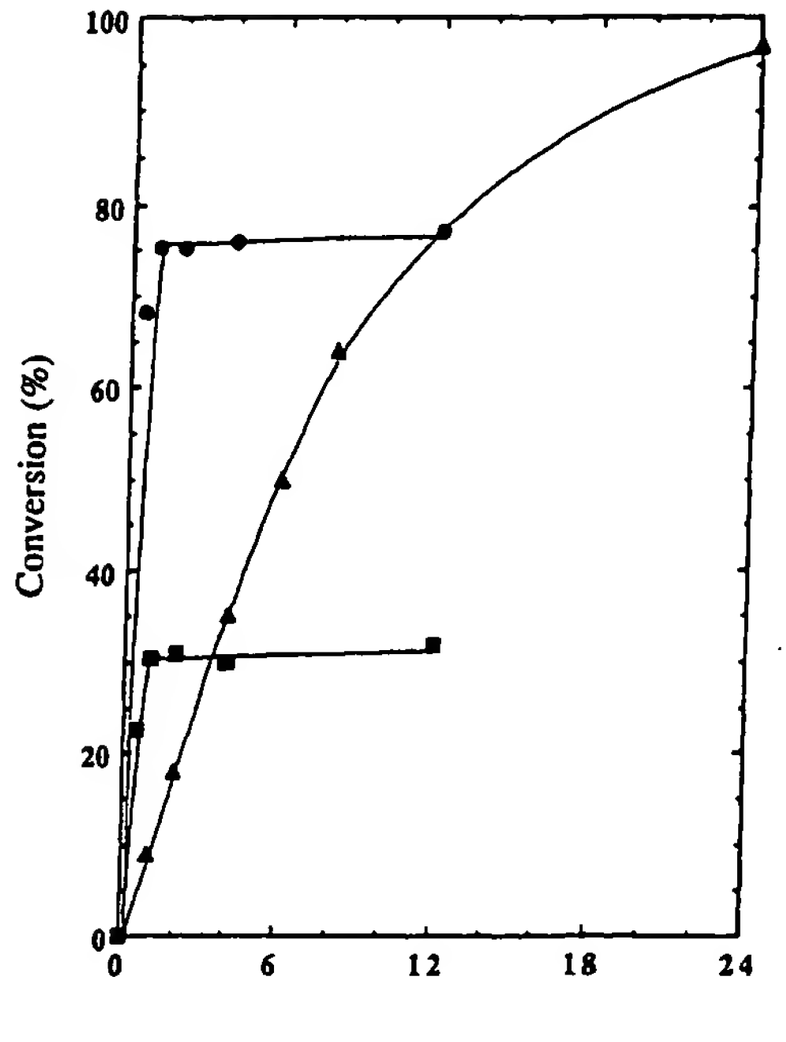

Figure 5. Ester formation by *Candida cylindracea* lipase: ▲, ethyl butyrate produced in *n*-heptane (Gillies et al., 1987); ●, octyl oleate produced in SC-CO_2 (this work); ■, ethyl oleate produced in SC-CO_2 (this work).

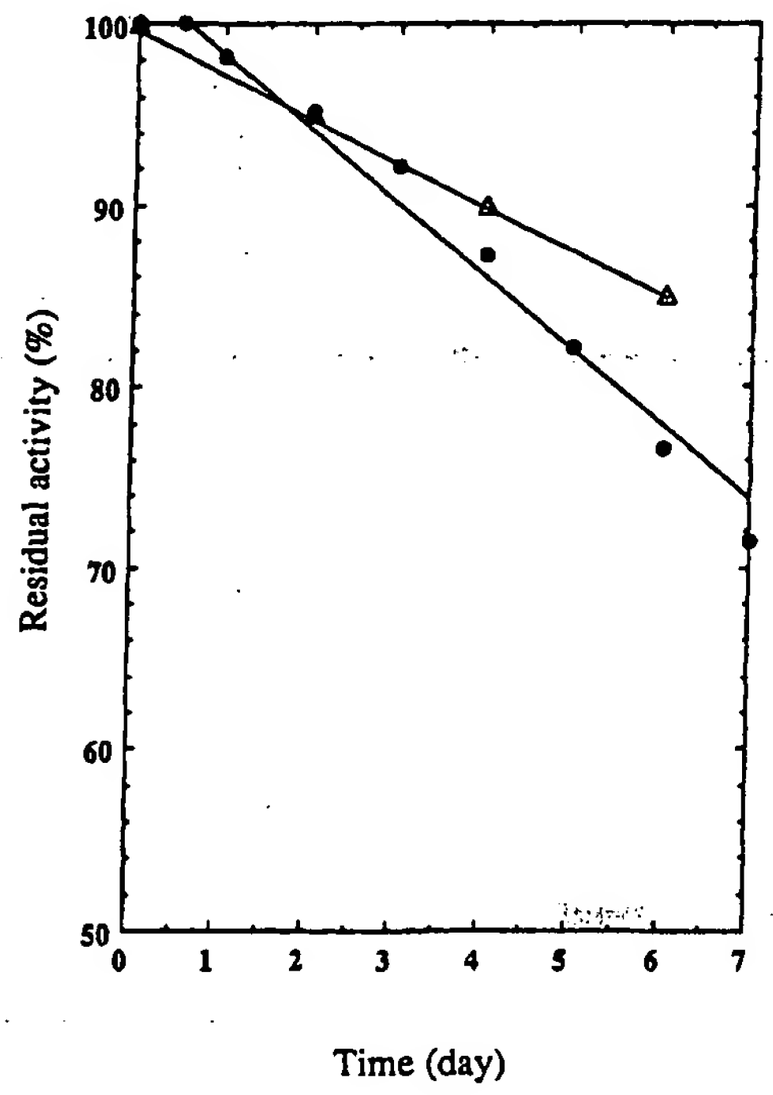

Figure 6. Stability of lipase in SC-CO_2: ▲, lipase from *Mucor miehei* (Marty et al., 1990); ●, lipase from *Candida cylindracea* (this work).

macroporous anionic resin beads (Marty et al., 1990) in SC-CO_2 at 13.6 MPa and 40 °C. The *Candida cylindracea* lipase exhibits about 75% activity after 7 days. Marty et al. (1990) found that the residual activity is about 85–90% after 6 days in either SC-CO_2 or *n*-hexane. It appears that the use of SC-CO_2 at high pressure does not modify the stability of either lipase. The difference in denaturation trends shown in Figure 6 could be due to the lipase type or the immobilization support.

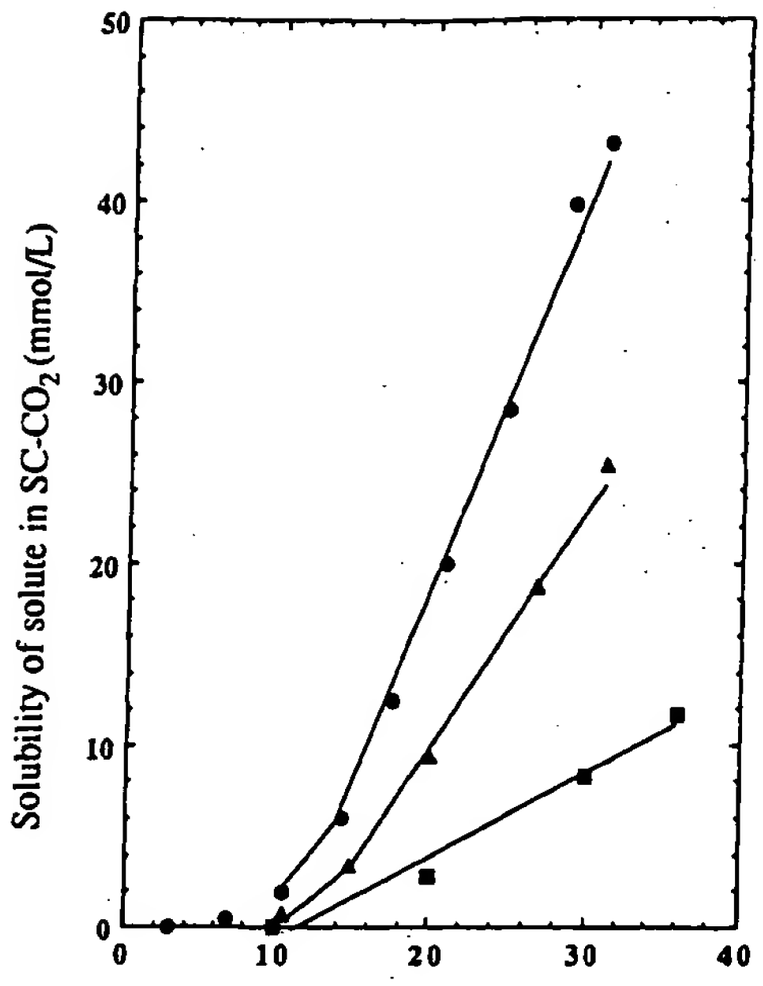

Figure 7. Solubilities of AMF, CNO, and a 1:1 mixture of AMF with CNO in SC-CO_2: ●, AMF (Yu et al., 1992); ■, CNO (Fattori et al., 1988); ▲, 1:1 mixture of AMF with CNO (this work).

Table I. Fatty Acid Composition (% by weight) Before and After Enzymatic Esterification with Ethanol in SC-CO_2

fatty acid	before	after	change
	Saturated		
C4:0	1.79	4.38	+145
C6:0	1.03	2.13	+106
C8:0	1.00	2.32	+131
C10:0	2.57	5.64	+120
C12:0	3.42	4.42	+29
C14:0	9.02	10.49	+16
C16:0	29.19	26.38	−10
C18:0	15.58	13.27	−15
C4:0–C8:0	3.82	8.83	+131
C10:0–C14:0	15.01	20.55	+37
C16:0–C18:0	44.76	39.65	−11
	Unsaturated		
C14:1	0.37	0.77	+107
C16:1	1.36	1.41	+3
C18:1	31.02	25.64	−17
C18:2	2.80	2.39	−15
C18:3	0.84	0.76	−10
C14:1–C18:3	36.40	30.97	−15

Esterification of Fatty Acids of AMF + Ethanol in SC-CO_2. The reaction conditions for esterification of fatty acids from milk fat with ethanol were the same as for oleic acid: 13.6 MPa at 40 °C. Table I compares the fatty acid composition before and after enzymatic esterification. The conversion of the short-chain fatty acids (C_4–C_8) was measurably higher than the conversion of the long-chain fatty acids.

Under these conditions, the reaction time for the enzymatic esterification of fatty acids from milk fat was approximately 1 h in SC-CO_2. However, the same reaction using the organic solvent ethanol required 120 h (Kanisawa, 1983). In Kanisawa's experiment, this reaction was carried out at 26 °C by adding 0.4% lipase (30 000 units/g) and 10% ethanol in the mixtures of fatty acids from butter fat.

Interesterification of AMF + CNO in SC-CO_2. The solubilities of AMF, CNO, and AMF + CNO mixtures in SC-CO_2 are given in Figure 7. The solubility of a 1:1 (wt) mixture of AMF and CNO in SC-CO_2 was measured in the same way as described previously (Yu et al., 1992). Although the optimum solubility of fats and oils probably occurs at higher pressure, the interesterification of AMF with CNO was carried out at 40 °C and 30 MPa because of the high cost of higher pressure equipment.

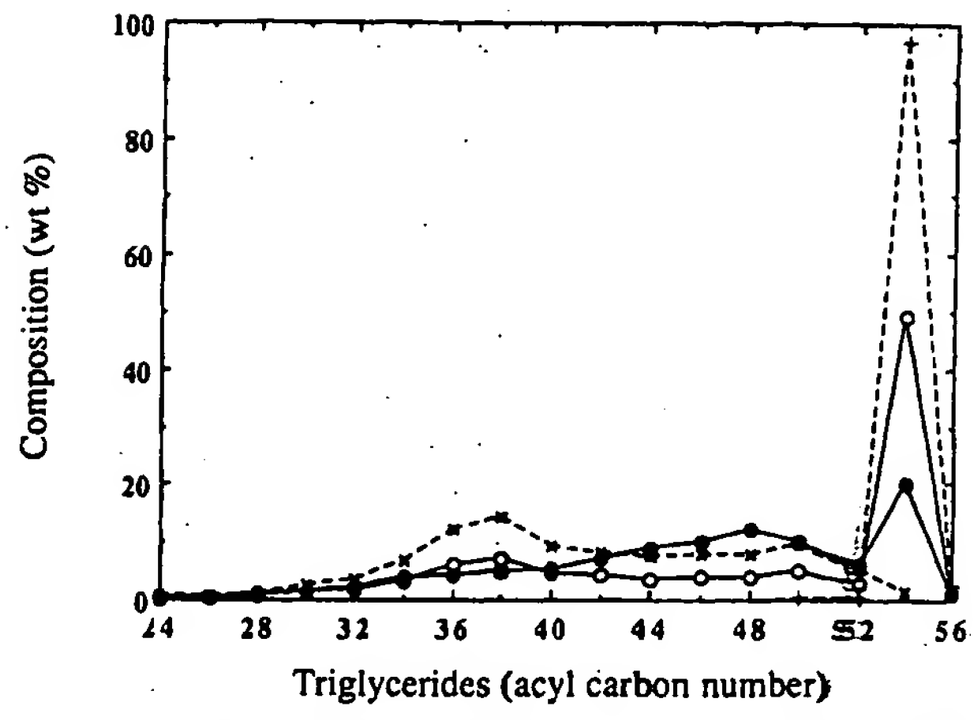

Figure 8. Triglyceride composition profiles (×, AMF; +, CNO) before (○) and after (●) enzymatic interesterification.

The carbon numbers of triglycerides formed by enzymatic interesterification of a 1:1 mixture of AMF and CNO are shown in Figure 8. The starting mixture contained predominantly C_{52}–C_{56} triglycerides from CNO and C_{32}–C_{38} triglycerides from AMF. The triglycerides formed by the lipase-catalyzed reaction of a 1:1 mixture of AMF and CNO contained mainly C_{42}–C_{50} and C_{54}.

Conclusions

This article describes the synthesis of ethyl esters by *Candida cylindracea* lipase in SC-CO_2. This lipase exhibits a high conversion of ethyl esters for short-chain fatty acids and, therefore, preferentially synthesizes them. The interesterification of fats and oils can be performed using the same immobilized lipase with the same water content as used for the ethyl oleate synthesis, but it requires a higher pressure due to the lower substrate solubility in SC-CO_2.

Acknowledgment

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Registry No. C18:0, 112-80-1; C18:0 ethyl ester, 111-62-6; C18:0 octyl ester, 32953-65-4; CO_2, 124-38-9; triacylglycerol lipase, 9001-62-1; ethanol, 64-17-5.